

USC3/35067

269 M  
1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 September 2003 (04.09.2003)

PCT

(10) International Publication Number  
WO 03/072705 A2

(51) International Patent Classification<sup>7</sup>: C12N (74) Agent: TERPSTRA, Anita, J.; McDonnell Boehnen Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).

(21) International Application Number: PCT/US03/03662 (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 6 February 2003 (06.02.2003) (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English (26) Publication Language: English

(30) Priority Data:

60/358,580	20 February 2002 (20.02.2002)	US
60/363,124	11 March 2002 (11.03.2002)	US
60/386,782	6 June 2002 (06.06.2002)	US
60/406,784	29 August 2002 (29.08.2002)	US
60/408,378	5 September 2002 (05.09.2002)	US
60/409,293	9 September 2002 (09.09.2002)	US
60/411,275	17 September 2002 (17.09.2002)	US
60/440,129	15 January 2003 (15.01.2003)	US



(71) Applicant (for all designated States except US): RI-  
BOZYME PHARMACEUTICALS, INC. [US/US];  
2950 Wilderness Place, Boulder, CO 80301 (US).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/072705 A2

(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF CYCLIN D1 GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siRNA)

(57) Abstract: The present invention concerns methods and reagents useful in modulating cyclin (e.g., cyclin D1) gene expression in a variety of applications, including use in therapeutic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siRNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against cyclin D1 gene expression. The molecules are useful in the treatment of cancer.

11503135067

**RNA INTERFERENCE MEDIATED INHIBITION OF CYCLIN D1 GENE  
EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)**

This invention claims the benefit of Thompson, USSN 60/411,275 filed September 17, 2002, of Beigelman USSN 60/358,580 filed February 20, 2002, of Beigelman USSN 5 60/363,124 filed March 11, 2002, of Beigelman USSN 60/386,782 filed June 6, 2002, of Beigelman USSN 60/406,784 filed August 29, 2002, of Beigelman USSN 60/408,378 filed September 5, 2002, of Beigelman USSN 60/409,293 filed September 9, 2002, of Beigelman USSN 60/427,467 filed November 19, 2002, and of Beigelman USSN 60/440,129 filed 10 January 15, 2003. These applications are hereby incorporated by reference herein in their entireties, including the drawings.

Field Of The Invention

The present invention concerns compounds, compositions, and methods for the study, diagnosis, and treatment of conditions and diseases that respond to the modulation of cyclin D1 gene expression and/or activity. The present invention also concerns compounds, 15 compositions, and methods relating to conditions and diseases that respond to the modulation of expression or activity of genes involved in the cyclin D1 pathway. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA 20 interference (RNAi) against cyclin D1 genes.

Background Of The Invention

The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

25 RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-

transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358).

5 Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to

10 be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short

15 pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that

20 are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the

25 siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001,

*Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877) has revealed certain requirements for siRNA length, structure, chemical composition, 5 and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be 10 tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA 15 duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309).

Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two -nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of 20 the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). In addition, Elbashir *et al.*, *supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA may include modifications 25 to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 30 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order

to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in siRNA molecules.

5        Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had substantial 10 decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease 15 in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish 20 reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

25        The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila* *in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure

genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of 5 particular genes in mammalian cells using certain dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain dsRNA molecules into cells for use in inhibiting gene expression. Plaetinck *et al.*, International PCT Publication No. WO 00/01846, describe certain methods for identifying 10 specific genes responsible for conferring a particular phenotype in a cell using specific dsRNA molecules. Mello *et al.*, International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Deschamps Depaillette *et al.*, International PCT Publication No. WO 99/07409, describe specific 15 compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.*, International PCT Publication No. 99/53050, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.*, International PCT Publication No. WO 01/49844, describe specific DNA constructs for use in facilitating gene silencing in targeted organisms.

Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, describe specific chemically-modified 20 siRNA constructs targeting the unc-22 gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.*, International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al.*, International PCT Publication No. WO 25 01/53475, describe certain methods for isolating a *Neurospora* silencing gene and uses thereof. Reed *et al.*, International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.*, International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as 30 Parkinson's Disease models using certain dsRNAs. Deak *et al.*, International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may

be related to RNAi in *Drosophila*. Arndt *et al.*, International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.*, International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.*, International PCT Publication No. WO 00/63364, and Satishchandran *et al.*, International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain dsRNAs. Echeverri *et al.*, International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.*, International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using RNAi. Graham *et al.*, International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.*, US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain siRNA constructs that mediate RNAi.

15

## SUMMARY OF THE INVENTION

This invention relates to compounds, compositions, and methods useful for modulating cyclin gene (e.g., cyclin D1) expression by RNA interference (RNAi) using small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression cyclin genes, such as cyclin D1. A siNA of the invention can be unmodified or chemically-modified. A siNA of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable modulating cyclin gene expression or activity in cells by RNA inference (RNAi). The use of chemically-modified siNA improves various properties of native siNA molecules through increased resistance to nuclease degradation *in vivo* and/or

through improved cellular uptake. Further, contrary to earlier published studies, siNA having multiple chemical modifications retains its RNAi activity. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

5        In one embodiment, the invention features one or more siNA molecules and methods that independently or in combination modulate the expression of gene(s) encoding cyclin proteins, such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in **Table I**, referred to herein generally as cyclin D1 (also known as CCND1). The description below of the various aspects and embodiments of the  
10      invention is provided with reference to exemplary cyclin D1 gene. However, the various aspects and embodiments are also directed to other other genes involved in cyclin D1 regulatory pathways. As such, the various aspects and embodiments of this invention are also directed to other genes that are involved in cyclin or cyclin D1 pathways of gene expression or activity. Those additional genes can be analyzed for target sites using the  
15      methods described herein for cyclin D1 genes. Thus, the inhibition and the effects of such inhibition of the other genes can be determined as described herein.

In one embodiment, the invention features a siNA molecule that down-regulates expression of a cyclin D1 gene, for example, wherein the cyclin D1 gene comprises cyclin D1 encoding sequence.

20        In one embodiment, the invention features a siNA molecule having RNAi activity against cyclin D1 RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having cyclin D1 encoding sequence, such as those sequences having GenBank Accession Nos. shown in **Table I**. Chemical modifications as shown in **Table IV** or otherwise described herein can be applied to any siNA construct of the invention.

25        In another embodiment, the invention features a siNA molecule having RNAi activity against a cyclin D1 gene, wherein the siNA molecule comprises nucleotide sequence complementary to nucleotide sequence of a cyclin D1 gene, such as those cyclin D1 sequences having GenBank Accession Nos. shown in **Table I**. In another embodiment, a

5 siNA molecule of the invention includes nucleotide sequence that can interact with nucleotide sequence of a cyclin D1 gene and thereby mediate silencing of cyclin D1 gene expression, for example, wherein the siNA mediates regulation of cyclin D1 gene expression by cellular processes that modulate the chromatin structure of the cyclin D1 gene and prevent transcription of the cyclin D1 gene.

10 In another embodiment, the invention features a siNA molecule comprising nucleotide sequence, for example, nucleotide sequence in the antisense region of the siNA molecule, that is complementary to a nucleotide sequence or portion of sequence of a cyclin D1 gene. In another embodiment, the invention features a siNA molecule comprising a region, for example, the antisense region of the siNA construct, complementary to a sequence or portion 15 of sequence comprising a cyclin D1 gene sequence.

15 In one embodiment, the antisense region of cyclin D1 siNA constructs can comprise a sequence complementary to sequence having any of SEQ ID NOs. 1-239 or 479-482. In another embodiment, the antisense region can comprise sequence having any of SEQ ID NOs. 240-478, 487-490, 495-498, 503-506, 521, 523, 525, 527, 529 or 530. In another embodiment, the sense region of cyclin D1 constructs can comprise sequence having any of SEQ ID NOs. 1-239, 479-486, 491-494, 499-502, 520, 522, 524, 526, or 528. The sense region can comprise a sequence of SEQ ID NO. 509 and the antisense region can comprise a sequence of SEQ ID NO. 510. The sense region can comprise a sequence of SEQ ID NO. 20 511 and the antisense region can comprise a sequence of SEQ ID NO. 512. The sense region can comprise a sequence of SEQ ID NO. 513 and the antisense region can comprise a sequence of SEQ ID NO. 514. The sense region can comprise a sequence of SEQ ID NO. 515 and the antisense region can comprise a sequence of SEQ ID NO. 516. The sense region can comprise a sequence of SEQ ID NO. 517 and the antisense region can comprise a sequence of SEQ ID NO. 518. The sense region can comprise a sequence of SEQ ID NO. 25 515 and the antisense region can comprise a sequence of SEQ ID NO. 519.

The sequences shown in SEQ ID NOs: 1-530 are not limiting. A siNA molecule of the invention can comprise any contiguous cyclin D1 sequence (e.g., about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25 contiguous cyclin D1 nucleotides).

In another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in Table I. Chemical modifications as shown in Table IV or otherwise described herein can be applied to any siNA construct of the invention.

In one embodiment of the invention a siNA molecule comprises an antisense strand having about 19 to about 29 nucleotides, wherein the antisense strand is complementary to a RNA sequence encoding a cyclin D1 protein, and wherein said siNA further comprises a sense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences with at least about 19 complementary nucleotides.

In another embodiment of the invention an siNA molecule of the invention comprises an antisense region having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a cyclin D1 protein, and wherein said siNA further comprises a sense region having about 19 to about 29 nucleotides, wherein said sense region and said antisense region comprise a linear molecule with at least about 19 complementary nucleotides.

In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a cyclin D1 gene or a portion thereof.

In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein. The siRNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a cyclin D1 gene or a portion thereof.

In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a cyclin D1 gene. Because cyclin genes as a group typically share some degree of sequence homology with each other, siNA molecules can be designed to target a class of cyclin genes or alternately specific cyclin genes by 5 selecting sequences that are either shared amongst different cyclin targets or that are alternately unique for a specific cyclin target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of cyclin encoding RNA sequence having homology between several cyclin genes so as to target several cyclin genes with one siNA molecule. In another embodiment, the siNA molecule can be designed to target a 10 sequence that is unique to a specific cyclin RNA sequence due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid molecules. In another embodiment, the siNA molecules of the invention consist of duplexes containing 15 about 19 base pairs between oligonucleotides comprising about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24 or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplexes with overhanging ends of about 1-3 (e.g., about 1, 2, or 3) nucleotides, for example about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs.

20 In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for cyclin D1 expressing nucleic acid molecules, such as RNA encoding a cyclin D1 protein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 25 "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish *et al.*, *supra*, applicant demonstrates that multiple

(greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to 5 improve *in vitro* or *in vivo* characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 10 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification 15 can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

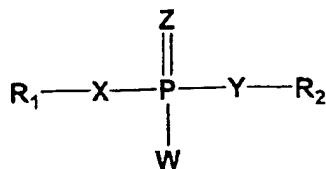
In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered 20 exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the 25 nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or

delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

The antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. The antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. The 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. The 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. The 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding cyclin D1 and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense regions. The siNA molecule can comprise a single strand having complementary sense and antisense regions.

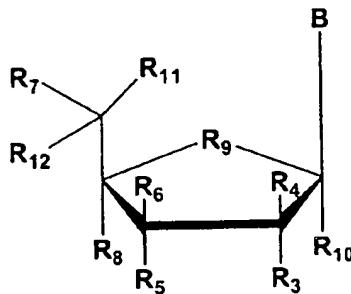
In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:



wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, 5 alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl, and wherein W, X, Y, and Z are optionally not all O.

The chemically-modified internucleotide linkages having Formula I, for example wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the 10 antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about one to about five or more (e.g., about 1, 2, 3, 4, 5, or more) 15 chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another 20 non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having 25 internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

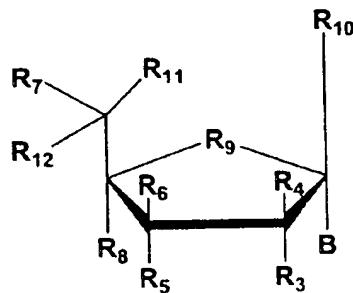


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, aminoalkyl, aminoacid, aminoacyl, ONH<sub>2</sub>, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I; R9 is O, S, CH<sub>2</sub>, S=O, CHF, or CF<sub>2</sub>, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about one to about

five or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about one to about five or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



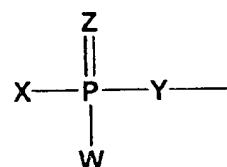
wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, aminoalkyl, aminoacid, aminoacyl, ONH<sub>2</sub>, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I; R9 is O, S, CH<sub>2</sub>, S=O, CHF, or CF<sub>2</sub>, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other

non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the 5 antisense strand, or both strands. The siNA molecules of the invention can comprise one or 10 more chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about one to about five or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non- 15 nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about one to about five or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

15 In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

20 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or alkylhalo; and wherein W, X, Y and Z are not all O.

In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1-3 (e.g., about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about one to about four (e.g., about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about one to about five or more (e.g., about 1, 2, 3, 4, 5, or more) consecutive

phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a siNA molecule, wherein the sense strand 10 comprises one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a 15 terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base 20 modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 25 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about one to about five, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) universal

base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about one to about five or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more)

5 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy,

10 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about one to about five or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about one to about ten or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap

molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about one to about five or more, specifically about 1, 2, 3, 4, 5 or more 5 phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about one to about five or more, 10 specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for 15 example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about one to about five, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

20 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about one to about five, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages in each strand of the siNA molecule.

In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, 25 or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or

more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

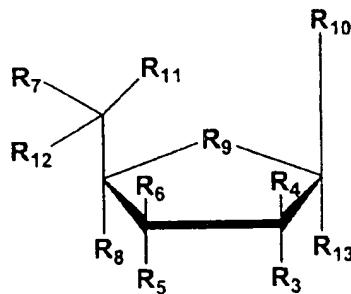
In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, 5 wherein each strand is about 18 to about 27 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27) nucleotides in length, wherein the duplex has about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be 10 chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (e.g., about 36, 40, 45, 50, 55, 60, 65, or 15 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that 20 is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear 25 hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60,

65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I—VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

In another embodiment, a circular siNA molecule of the invention contains two loop 10 motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

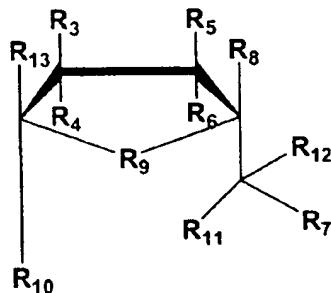
15 In one embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



20 wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, aminoalkyl, aminoacid, aminoacyl, ONH<sub>2</sub>, O-aminoalkyl, O-aminoacid, O-aminoacyl,

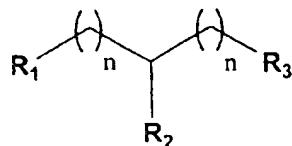
heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2.

In one embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound 5 having Formula VI:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-O-SH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention.

15 In another embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is 20 independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3,

OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO<sub>2</sub>, NO<sub>2</sub>, N3, NH<sub>2</sub>, aminoalkyl, aminoacid, aminoacyl, ONH<sub>2</sub>, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, 5 polyalkylamino, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n = 1, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a 10 double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in Figure 10).

In another embodiment, a moiety having any of Formula V, VI or VII of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. 15 For example, a moiety having Formula V, VI or VII can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

In another embodiment, a siNA molecule of the invention comprises an abasic residue 20 having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example 25 at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In another embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In one embodiment, the invention features a chemically-modified short interfering 5 nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any 10 (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering 15 nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any 20 (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering 25 nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and

wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

5 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a 10 plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are 15 present in said antisense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

25 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g.,

wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siRNAs are shown in **Figures 4 and 5 and Tables III and IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siRNA) molecule of the invention capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the siRNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine

nucleotides present in the sense region are purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region 5 optionally further comprising a 3'-terminal overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the siNA comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides 10 are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or 15 both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples 20 of these chemically-modified siNAs are shown in **Figures 4 and 5 and Tables III and IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against cyclin D1 inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and for example where one or more purine nucleotides present in the sense region are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid 25 (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl 30 nucleotides.

nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and wherein inverted deoxy abasic modifications are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about one to about four (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages.

In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also 30 optionally in the sense and/or both antisense and sense strands, comprise modified

nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically 5 modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O,4'- 10 C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against cyclin 15 D1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached 20 at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a 25 conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a poly ethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the 30 instant invention that can be attached to chemically-modified siNA molecules are described

in Vargeese *et al.*, U.S. Serial No. 60/311,865, incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of  $\geq 2$  nucleotides in length, for example 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In yet another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.

In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. polyethylene glycols such as those having between 2 and

100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma *et al.*, *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand *et al.*, *Nucleic Acids Res.* 1990, 18:6353; McCurdy *et al.*, *Nucleosides & Nucleotides* 1991, 10:287; Jschke *et al.*, *Tetrahedron Lett.* 1993, 34:301; Ono *et al.*, *Biochemistry* 1991, 30:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, 5 all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, 10 cytosine, uracil or thymine, for example at the C1 position of the sugar.

15

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides. All positions within 20 the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA 25 molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-

terminal phosphate group (e.g., a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 19 to about 29 nucleotides. In yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all 5 the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the 10 siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine 15 nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further 20 comprising about one to about four (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

25 In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides

are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about one to about four (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about one to about four (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the 5 siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a 10 plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about one to about four (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal 15 nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics 20 similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation 25 while at the same time maintaining the capacity to mediate RNAi.

In one embodiment, the invention features a method for modulating the expression of a cyclin D1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence

complementary to RNA of the cyclin D1 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the cyclin D1 gene in the cell.

In one embodiment, the invention features a method for modulating the expression of a cyclin D1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 gene and wherein the sense strand sequence of the siNA is identical to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the cyclin D1 gene in the cell.

10 In another embodiment, the invention features a method for modulating the expression of more than one cyclin D1 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the cyclin 15 D1 genes in the cell.

20 In another embodiment, the invention features a method for modulating the expression of more than one cyclin D1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 gene and wherein the sense strand sequence of the siNA is identical to the sequence of the target RNA; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the cyclin D1 genes in the cell.

25 In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the cyclin D1 gene in the tissue explant. In another

embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the cyclin D1 gene in that organism.

In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 gene and wherein the sense strand sequence of the siNA is identical to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the cyclin D1 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the cyclin D1 gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one cyclin D1 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the cyclin D1 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the cyclin D1 genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the cyclin D1 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one cyclin D1 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of the cyclin D1 genes; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the cyclin D1 genes in the organism.

In one embodiment, the invention features a method for modulating the expression of a cyclin D1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the cyclin D1 gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one cyclin D1 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) contacting the siNA molecule with a cell in vitro or in vivo under conditions suitable to modulate the expression of the cyclin D1 genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) contacting the siNA molecule with a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the cyclin D1 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the cyclin D1 gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one cyclin D1 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the cyclin D1 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the cyclin D1 genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the cyclin D1 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one cyclin D1 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the cyclin D1 gene; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the cyclin D1 genes in the organism.

In one embodiment, the invention features a method of modulating the expression of a cyclin D1 gene in an organism comprising contacting the organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the cyclin D1 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one cyclin D1 gene in an organism, comprising contacting the organism with

one or more siNA molecules of the invention under conditions suitable to modulate the expression of the cyclin D1 genes in the organism.

The siNA molecules of the invention can be designed to inhibit target (cyclin D1) gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the 5 siNA molecules of the invention are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family 10 of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of 15 pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the 20 invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

In another embodiment, the siNA molecules of the invention are used to target 25 conserved sequences corresponding to a gene family or gene families such as cyclin genes. As such, siNA molecules targeting multiple cyclin targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions

toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, the progression and/or maintenance of cancer.

In one embodiment, siNA molecule(s) and/or methods of the invention are used to inhibit the expression of gene(s) that encode RNA referred to by Genbank Accession, for 5 example cyclin D1 genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example Genbank Accession Nos. shown in Table I.

In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the 10 target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another 15 embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or 20 transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of  $4^N$ , where N represents the number of base paired nucleotides in each of the siNA construct strands (e.g. for a siNA construct having 21 nucleotide sense and antisense strands with 19 25 base pairs, the complexity would be  $4^{19}$ ); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target cyclin D1 RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (e.g., about

19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 7 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of cyclin D1 RNA are analyzed for detectable levels of 5 cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target cyclin D1 RNA sequence. The target cyclin D1 RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

10 In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have 15 strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is 20 expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

25 By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the

background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising siNA molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In yet another embodiment, the invention features a method for reducing or preventing tissue rejection in a subject comprising administering to the subject a composition of the invention under conditions suitable for the reduction or prevention of tissue rejection in the subject.

In another embodiment, the invention features a method for validating a cyclin D1 gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a cyclin D1 target gene; (b) introducing the siNA molecule into a cell, tissue, or organism under conditions suitable for modulating expression of the cyclin D1 target gene in the cell, tissue, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, or organism.

In another embodiment, the invention features a method for validating a cyclin D1 gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a cyclin D1 target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the cyclin D1 target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

By "biological system" is meant, material, in a purified or unpurified form, from biological or non-biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi activity. The term "biological system" can include a cell, tissue, or 5 organism, or extract thereof. As such, the term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (e.g., siNA). Such detectable changes include but are not limited to changes in shape, size, proliferation, 10 motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change can also include expression of reporter genes/molecules such as Green Fluorescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

In one embodiment, the invention features a kit containing a siNA molecule of the 15 invention, which can be chemically-modified, that can be used to modulate the expression of a cyclin D1 target gene in a cell, tissue, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one cyclin D1 target gene in a cell, tissue, or organism.

20 In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

25 In one embodiment, the synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In

yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions using an alkylamine base such as methylamine. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example using acidic conditions.

In a further embodiment, the method for siNA synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of

the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

In another embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of the siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety than can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

In another embodiment, the invention features a method for making a double-stranded siNA molecule in a single synthetic process, comprising: (a) synthesizing an oligonucleotide

having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

In another embodiment, the method of synthesis of siNA molecules of the invention 10 comprises the teachings of Scaringe *et al.*, US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications, for example one or more chemical modifications having any of Formulae I-VII or any 15 combination thereof that increases the nuclease resistance of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased 20 nuclease resistance.

In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

25 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under

conditions suitable for isolating siNA molecules having increased binding affinity between the sense and antisense strands of the siNA molecule.

5 In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

10 In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

15 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence, comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

20 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence, comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

25 In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of

generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

In another embodiment, the invention features a method for generating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of 5 generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating 10 additional endogenous siNA molecules having sequence homology to the chemically-modified siNA molecule.

In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against cyclin D1 in a cell, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule 15 and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against cyclin D1 comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA 20 molecules having improved RNAi activity.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a cyclin D1 target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA. 25

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a cyclin D1 target DNA comprising (a)

introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target DNA.

5 In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

10 In another embodiment, the invention features a method for generating siNA molecules against cyclin D1 with improved cellular uptake, comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

15 In one embodiment, the invention features siNA constructs that mediate RNAi against cyclin D1, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.*, U.S. Serial No. 60/311,865 incorporated by reference herein.

20 In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability, comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such conjugates can include ligands for cellular receptors such as peptides derived from naturally occurring protein ligands protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins; and other co-factors such as folate and N-acetylgalactosamine, polymers such as polyethyleneglycol (PEG), phospholipids, polyamines such as spermine or spermidine, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such 5 excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule, and (b) assaying the 10 siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

15 The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include the siNA and a vehicle that promotes introduction of the siNA. Such a kit can also include instructions to allow a user of the kit to practice the invention.

20 The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.*, 25 2001, *Nature*, 411, 494-498; and Kreutzer *et al.*, International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.*, International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO

01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.*, International PCT Publication No. WO 00/44914; Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus *et al.*, 2002, *RNA*, 8, 842-850; Reinhart *et al.*, 2002, *Gene & Dev.*, 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). Nonlimiting examples of siNA molecules of the invention are shown in **Figures 4-6, and Tables II, III, and IV** herein. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises 5 nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from 10 two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each 15 strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA is assembled from a single oligonucleotide, where 20 the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the 25 sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense 30 region having nucleotide sequence corresponding to the target nucleic acid sequence or a

portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, 5 where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.*, 2002, *Cell.*, 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain 10 embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically- 15 modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any 20 ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide 25 positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides ""siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering 30

modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, or epigenetics. For example, siNA molecules of the invention 5 can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and 10 Hall *et al.*, 2002, *Science*, 297, 2232-2237).

By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the 15 modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit" it is meant that the activity of a gene expression product or level of RNAs or equivalent RNAs encoding one or more gene products is reduced below that observed in the absence of the nucleic acid molecule of the invention. In one embodiment, inhibition 20 with a siNA molecule preferably is below that level observed in the presence of an inactive or attenuated molecule that is unable to mediate an RNAi response. In another embodiment, inhibition of gene expression with the siNA molecule of the instant invention is greater in the presence of the siNA molecule than in its absence.

By "gene" or "target gene" is meant, a nucleic acid that encodes an RNA, for example, 25 nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or

fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts.

By "cyclin D1" is meant, any cyclin (e.g., cyclin D1) polypeptide, protein and/or a 5 polynucleotide encoding a cyclin (e.g., cyclin D1) protein (such as polynucleotides referred to by Genbank Accession number in Table I or any other cyclin transcript derived from a cyclin (e.g., cyclin D1) gene).

By "cyclin D1 protein" is meant, any cyclin (e.g., cyclin D1) peptide or protein or a component thereof, wherein the peptide or protein is encoded by a cyclin (e.g., cyclin D1) 10 gene or having cyclin (e.g., cyclin D1) activity.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "sense region" is meant a nucleotide sequence of a siNA molecule having 15 complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a 20 siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with 25 another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy

for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.*, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The siNA molecules of the invention represent a novel therapeutic approach to treat a broad spectrum of oncology and proliferation related indications and conditions, including but not limited cancer, such as breast cancer, cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, multiple myeloma, ovarian cancer, melanoma, colorectal cancer, prostate cancer, and any other indications that can respond to the level of cyclin D1 in a cell or tissue.

In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 18 to about 24 nucleotides in length, in specific embodiments about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 17 to about 23 base pairs (e.g., about 17, 18, 19, 20, 21, 22 or 23). In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (e.g., about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (e.g., 38, 39, 40, 41, 42, 43 or 44) nucleotides in length and comprising about 16 to about 22 (e.g., about 16, 17, 18, 19, 20, 21 or 22) base pairs. Exemplary siNA molecules of the invention are shown in **Tables II**. Exemplary synthetic siNA molecules of the invention are shown in **Tables III and IV** and/or **Figures 4-5**.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or 5 eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. 10 The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in Tables II-III and/or Figures 4-5. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. 15 Furthermore, the chemical modification constructs described in Table IV can be applied to any siNA sequence of the invention.

In another aspect, the invention provides mammalian cells containing one or more siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

20 By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a  $\beta$ -D-ribofuranose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the 25 addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-

naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. In one embodiment, a subject is a mammal or mammalian cells. In another embodiment, a subject is a human or human cells.

The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

10 The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 15 2001, *Nucleic Acids Research*, 29, 2437-2447).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

20 The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein (e.g., cancer). For example, to treat a particular disease or condition, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

25 In a further embodiment, the siNA molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic

agents to treat a disease or condition. Non-limiting examples of other therapeutic agents that can be readily combined with a siNA molecule of the invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic 5 and/or inorganic compounds including metals, salts and ions.

In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain 10 sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul *et al.*, 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee *et al.*, 2002, *Nature Biotechnology*, 19, 500; and Novina *et al.*, 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725.

15 In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. shown in Table I.

20 In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from 25 transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and

persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by 5 intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

10 Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are 15 synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on 20 the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

25 Figure 2 shows a MALDI-TOV mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated

from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

Figure 3 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by 5 RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directly into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex 10 or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

Figure 4A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, 15 cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs.

Figure 4A: The sense strand comprises 21 nucleotides having four phosphorothioate 5'- and 3'-terminal internucleotide linkages, wherein the two terminal 3'-nucleotides are 20 optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally 25 complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and four 5'-terminal phosphorothioate internucleotide linkages and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

**Figure 4C:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, 15 deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, 20 universal bases, or other chemical modifications described herein.

**Figure 4D:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'- nucleotides are optionally complementary to the target RNA sequence, and having one 3'-

terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4E: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4F: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of the invention.

**Figure 5A-F** shows non-limiting examples of specific chemically-modified siNA sequences of the invention. A-F applies the chemical modifications described in **Figure 4A-F** to a cyclin D1 siNA sequence.

**Figure 6** shows non-limiting examples of different siNA constructs of the invention.

5 The examples shown (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-  
10 nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 *in vivo* and/or *in vitro*. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 *in vivo* and/or *in vitro*, which can optionally  
15 utilize another biodegradable linker to generate the active siNA construct 1 *in vivo* and/or *in vitro*. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use *in vivo* or *in vitro* and/or *in vitro*.

**Figure 7A-C** is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siNA hairpin constructs.

20 **Figure 7A:** A DNA oligomer is synthesized with a 5'-restriction site (R1) sequence followed by a region having sequence identical (sense region of siNA) to a predetermined cyclin D1 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

25 **Figure 7B:** The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a siNA transcript having specificity for a cyclin D1 target sequence and having self-complementary sense and antisense regions.

5 **Figure 7C:** The construct is heated (for example to about 95°C) to linearize the sequence, thus allowing extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'- terminal nucleotide overhang results from the transcription, for example by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul *et al.*, 2002, *Nature Biotechnology*, 29, 505-508.

10 **Figure 8A-C** is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siNA constructs.

15 **Figure 8A:** A DNA oligomer is synthesized with a 5'-restriction (R1) site sequence followed by a region having sequence identical (sense region of siNA) to a predetermined cyclin D1 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

20 **Figure 8B:** The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

25 **Figure 8C:** The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

30 **Figure 9A-E** is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA.

35 **Figure 9A:** A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target nucleic

acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA.

**Figure 9B&C:** (Figure 9B) The sequences are pooled and are inserted into vectors such that (Figure 9C) transfection of a vector into cells results in the expression of the siNA.

5 **Figure 9D:** Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

**Figure 9E:** The siNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

10 **Figure 10** shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including (1) [3-3']-inverted deoxyribose; (2) deoxyribonucleotide; (3) [5'-3']-3'-deoxyribonucleotide; (4) [5'-3']-ribonucleotide; (5) [5'-3']-3'-O-methyl ribonucleotide; (6) 3'-glyceryl; (7) [3'-5']-3'-deoxyribonucleotide; (8) [3'-3']-deoxyribonucleotide; (9) [5'-2']-deoxyribonucleotide; and (10) [5-3']-dideoxyribonucleotide. In addition to modified and 15 unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any 20 combination thereof.

25 **Figure 11** shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-modifications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct is tested in an appropriate system (e.g. human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter

assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

5       Figure 12 shows a non-limiting example of reduction of cyclin D1 mRNA in A549 cells mediated by chemically-modified siNAs that target cyclin D1 mRNA. A549 cells were transfected with 0.25 ug/well of lipid complexed with 25 nM siNA. A siNA construct comprising ribonucleotides and 3'-terminal dithymidine caps (RPI#31009/31085) was compared to a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro 10 pyrimidine nucleotides and purine ribonucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31304/31305), which was also compared to a matched chemistry inverted control (RPI#31316/31317). In addition, 15 the siNA constructs were also compared to untreated cells, cells transfected with lipid and scrambled siNA constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in the figure, both siNA constructs show significant reduction of cyclin D1 RNA expression.

#### Mechanism of action of Nucleic Acid Molecules of the Invention

20       The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity *in vivo*; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to 25 siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of

ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which 10 is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA 15 in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III 20 enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) 25 from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle

of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably through cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences 5 (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237). As such, siRNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level 10 or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa 15 cells. Recent work in *Drosophila* embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are 20 most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the 25 siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309); however, siRNA

molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'- phosphorylation of siRNA constructs may occur *in vivo*.

#### Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using 5 automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The 10 simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, 15 for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides 20 makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2  $\mu$ mol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-fluoro nucleotides. Table V outlines 25 the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2  $\mu$ mol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60  $\mu$ L of 0.11 M = 6.6  $\mu$ mol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60  $\mu$ L of 0.25 M = 15  $\mu$ mol) can be used in each coupling

cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40  $\mu$ L of 0.11 M = 4.4  $\mu$ mol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40  $\mu$ L of 0.25 M = 10  $\mu$ mol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I<sub>2</sub>, 49 mM pyridine, 9% water in THF (PERSEPTIVE<sup>TM</sup>). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

15 Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H<sub>2</sub>O/3:1:1, vortexed and the supernatant is then 20 added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2  $\mu$ mol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step

for 2'-O-methylated nucleotides. Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2  $\mu$ mol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60  $\mu$ L of 0.11 M = 6.6  $\mu$ mol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60  $\mu$ L of 0.25 M = 15  $\mu$ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120  $\mu$ L of 0.11 M = 13.2  $\mu$ mol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120  $\mu$ L of 0.25 M = 30  $\mu$ mol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I<sub>2</sub>, 49 mM pyridine, 9% water in THF (PERSEPTIVE<sup>TM</sup>). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H<sub>2</sub>O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300  $\mu$ L of a solution of 1.5 mL N-methylpyrrolidinone, 750  $\mu$ L TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH<sub>4</sub>HCO<sub>3</sub>.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to rt. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at -20 °C 5 and then quenched with 1.5 M NH<sub>4</sub>HCO<sub>3</sub>.

For purification of the trityl-on oligomers, the quenched NH<sub>4</sub>HCO<sub>3</sub> solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 10 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including 15 but not limited to 96-well format.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; 20 Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which 25 is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well

platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al.*, *supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

20 Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see e.g., Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; 25 and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.*, U.S. Pat. No. 6,300,074; and Burgin *et al.*, *supra*; all of which are incorporated by reference herein). All of the above references describe various chemical

modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

5 There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-10 allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS*, 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al.* *Nature*, 1990, 344, 565-568; Pieken *et al.* *Science*, 1991, 253, 314-15 317; Usman and Cedergren, *Trends in Biochem. Sci.*, 1992, 17, 334-339; Usman *et al.* *International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.*, 1995, *J. Biol. Chem.*, 270, 25702; Beigelman *et al.*, *International PCT publication* No. WO 97/26270; Beigelman *et al.*, *U.S. Pat.* No. 5,716,824; Usman *et al.*, *U.S. Pat.* No. 5,627,053; Woolf *et al.*, *International PCT Publication* No. WO 98/13526; 20 Thompson *et al.*, *USSN 60/082,404* which was filed on April 20, 1998; Karpeisky *et al.* 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.*, 67, 99-134; and Burlina *et al.*, 1997, *Bioorg. Med. Chem.*, 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi in cells is not 25 significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or

more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention 10 encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed 15 to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically 20 active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the 25 invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified

nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 5 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, 10 for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active 15 molecules such as antibodies, hormones, antivirals, peptides, proteins, therapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the 20 pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, 25 COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules (e.g., siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated

long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by 5 introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

10 Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of 15 subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example on only the sense siNA strand, the antisense siNA strand, or 20 both siNA strands.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.*, U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or 25 localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap is selected from the group consisting of glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic

nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide 5 moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

In non-limiting examples, the 3'-cap is selected from a group consisting of glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-10 erythrophuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl 15 nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; 20 bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly 25 recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons.

The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub>, halogen, N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino or SH.

Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thieryl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, 5 Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & 10 Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, 15 *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' 20 position or their equivalents.

20 In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amide carbamate, carboxymethyl, acetamide, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see 25 Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic *et al.*, U.S. Pat. No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of  $\beta$ -D-ribo-furanose.

5 By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

10 In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH<sub>2</sub> or 2'-O- NH<sub>2</sub>, which may be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Pat. No. 5,672,695 and Matulic-Adamic *et al.*, U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

15 Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

#### Administration of Nucleic Acid Molecules

20 A siNA molecule of the invention can be adapted for use to treat cancer, including but not limited to breast cancer, cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, multiple myeloma, ovarian cancer, melanoma, colorectal cancer, prostate cancer, lung cancer, bladder cancer, esophageal cancer, glioblastoma, and 25 proliferative conditions such as restinosis, and other indications that can respond to the level of cyclin D1 in a cell or tissue, alone or in combination with other therapies. For example, a siNA molecule can comprise a delivery vehicle, including liposomes, for administration to a

subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 5 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can 10 be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins (see for example Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT 15 Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry *et al.*, 1999, *Clin. Cancer Res.*, 5, 2330-2337 20 and Barry *et al.*, International PCT Publication No. WO 99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

Thus, the invention features a pharmaceutical composition comprising one or more 25 nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (e.g., RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The 30 compositions of the present invention can also be formulated and used as tablets, capsules or

elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid 5 addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of 10 entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation 15 from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: 20 intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system 25 (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention 5 include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al.*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of 10 polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 15, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the 20 accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.* *Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by 25 extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 1995, 267, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in 30 tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT

Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

5 The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985),  
10 hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a  
15 disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent  
20 upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or

adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

5 Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable 10 excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or 15 they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the 20 active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in a mixture with excipients suitable 25 for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example

heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions 5 can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as 10 liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid

Dispersible powders and granules suitable for preparation of an aqueous suspension by 15 the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water 20 emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for 25 example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a

demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile 5 injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be 10 employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary 15 temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, 20 preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated 25 and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body

weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triantennary structures are bound with greater affinity than biantennary or monoantennary chains (Baenziger and Fiete, 1980, *Cell*, 22, 611-620; Connolly *et al.*, 1982, *J. Biol. Chem.*, 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannose-terminating glycoproteins or glycoconjugates (Ponpipom *et al.*, 1981, *J. Med. Chem.*, 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds

required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese *et al.*, USSN 60/311,865, filed August 13, 2001; and Matulic-Adamic *et al.*, USSN 60/362,016, 5 filed March 6, 2002.

Alternatively, certain siNA molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985, *Science*, 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci., USA* 83, 399; Scanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 10 3-15; Dropulic *et al.*, 1992, *J. Virol.*, 66, 1432-41; Weerasinghe *et al.*, 1991, *J. Virol.*, 65, 5531-4; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science*, 247, 1222-1225; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Good *et al.*, 1997, *Gene Therapy*, 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the 15 appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994, *J. Biol. Chem.*, 269, 25856.

20 In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.*, 1996, *TIG.*, 12, 510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adenovirus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based 25 constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed,

the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells *ex-planted* from a subject followed by reintroduction into the subject, or by any other means that would allow for 5 introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.* 12, 510).

In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary 10 strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows expression of the siNA molecule (see for example Paul *et al.*, 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee *et al.*, 2002, *Nature Biotechnology*, 19, 500; and Novina *et al.*, 2002, *Nature Medicine*, advance online 15 publication doi:10.1038/nm725).

In another aspect, the invention features an expression vector comprising: a) a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant 20 invention; wherein said sequence is operably linked to said initiation region and said termination region in a manner that allows expression and/or delivery of the siNA molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

25 Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA

polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber *et al.*, 1993, *Methods Enzymol.*, 217, 47-66; Zhou *et al.*, 1990, *Mol. Cell. Biol.*, 10, 4529-37). Several 5 investigators have demonstrated that nucleic acid molecules expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.*, 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisziewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. 10 U. S. A*, 90, 8000-4; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as siNA in cells (Thompson *et al.*, *supra*; Couture and Stinchcomb, 1996, *supra*; Noonberg *et al.*, 1994, 15 *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, U.S. Pat. No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of vectors for introduction 20 into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the siNA molecules of the invention, in a manner that allows expression of that siNA molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; and c) 25 a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid

sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame; and wherein the sequence is operably linked to the initiation region, the open reading frame and the termination region, in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment the 5 expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

10 In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame, and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the 15 termination region in a manner which allows expression and/or delivery of the siNA molecule.

#### Cyclin D1 biology and biochemistry

The proliferation of mammalian cells by extracellular signals occurs during the G1 phase of the cell cycle. During this period, growth-stimulatory and growth-inhibitory signals 20 transduced from the extracellular environment impinge on the cell cycle apparatus operating in the nucleus. This apparatus, composed of cyclins and their cyclin-dependent kinases, can respond by setting into motion an autonomous cell division program that carries the cells through S, G2 and M phases or, alternatively, by causing exit from the active cell cycle into the quiescent G0 state. Four mammalian G1 cyclins have been described to date: cyclins D1, 25 D2, D3 and cyclin E. The expression of cyclin D1 is rapidly induced following the exposure of cells to mitogens; and cyclin D1 levels rapidly decline after the mitogens have been removed. This, together with a very short half-life of the cyclin D1 protein permits rapid modulation of cyclin D1 levels in response to changes in extracellular environment (Sherr, 1994, *Cell*, 79, 551). Cyclin D1 was originally cloned as an oncogene responsible for

parathyroid adenomas (Motokura *et al.*, 1991, *Nature*, 350, 512). Subsequently, the aberrant expression of cyclin D1 was documented in several human malignancies. Most striking is the frequent involvement of cyclin D1 in human breast cancers.

Early studies reported cyclin D1 gene amplification in 10-15% of mammary carcinomas. When anti-cyclin D1 antibodies became available, it was discovered that cyclin D1 protein overexpression is found in the majority of human breast cancers (Bartkova *et al.*, 1994, *Int. J. Cancer*, 57, 353). The overexpression of cyclin D1 protein correlates with the presence of receptors for ovarian steroids on tumors cells and is usually linked with poor prognosis. Recent studies have demonstrated that overexpression of cyclin D1 distinguishes malignant breast carcinomas from premalignant breast lesions (Weinstat-Saslow *et al.*, 1995, *Nature Medicine*, 1, 1257). Consistent with the oncogenic role of cyclin D1 is the observation that transgenic mice engineered to overexpress this cyclin in breast tissue are prone to mammary adenocarcinomas (Wang *et al.*, 1994, *Nature*, 369, 669).

The use of small interfering nucleic acid molecules targeting cyclin D1 provides a class of novel therapeutic agents that can be used in the treatment of cancers or any other disease or condition that responds to modulation of cyclin D1 genes.

Examples:

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

20 Example 1: Tandem synthesis of siNA constructs

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

After completing a tandem synthesis of a siNA oligo and its complement in which the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex 5 in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this 10 dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example by using a C18 cartridge.

Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see Figure 1) or an equivalent cleavable linker. A non-limiting example of linker coupling 15 conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexafluorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting 20 oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M NH<sub>4</sub>H<sub>2</sub>CO<sub>3</sub>.

Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H<sub>2</sub>O, and 2 CV 50mM NaOAc. The sample is loaded and 25 then washed with 1 CV H<sub>2</sub>O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H<sub>2</sub>O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The

remaining TFA solution is removed and the column washed with H<sub>2</sub>O followed by 1 CV 1M NaCl and additional H<sub>2</sub>O. The siNA duplex product is then eluted, for example using 1 CV 20% aqueous CAN.

Figure 2 provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA construct only shows a single peak.

Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2: Identification of potential siNA target sites in any RNA sequence

The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using *in vitro* RNA cleavage assays, cell

culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient 5 reduction in target gene expression.

Example 3: Selection of siNA molecule target sites in a RNA

The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

1. The target sequence is parsed *in silico* into a list of all fragments or subsequences of a 10 particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.
2. In some instances the siNAs correspond to more than one target sequence; such would be 15 the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain 20 the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.
- 25 3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the

lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

4. The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.  
5
5. The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.
6. The ranked siNA subsequences can be further analyzed and ranked according to whether 10 they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.
7. The ranked siNA subsequences can be further analyzed and ranked according to whether 15 they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.
8. Four or five target sites are chosen from the ranked list of subsequences as described 20 above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see Tables II and III). If terminal TT residues are 25 desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

9. The siNA molecules are screened in an *in vitro*, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.

In an alternate approach, a pool of siNA constructs specific to a cyclin D1 target sequence is used to screen for target sites in cells expressing cyclin D1 RNA, such as HELA, parathyroid adenoma, or A549 cells. The general strategy used in this approach is shown in Figure 9. A non-limiting example of such a pool is a pool comprising sequences having sense sequences comprising SEQ ID NOs. 1-239, 479-486, 491-494, and/or 499-502 and antisense sequences comprising SEQ ID NOs. 240-478, 487-490, 495-498, and/or 503-506 respectively. A549, parathyroid adenoma, or HELA cells expressing cyclin D1 are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with cyclin D1 inhibition are sorted. The pool of siNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example Figure 7 and Figure 8). The siNA from cells demonstrating a positive phenotypic change (e.g., decreased proliferation, decreased cyclin D1 mRNA levels or decreased cyclin D1 protein expression), are sequenced to determine the most suitable target site(s) within the target cyclin D1 RNA sequence.

Example 4: cyclin D1 targeted siNA design

siNA target sites were chosen by analyzing sequences of the cyclin D1 RNA target and 20 optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA molecules as described in Example 3, or alternately by using an *in vitro* siNA system as described in Example 6 herein. siNA molecules were designed that could bind each target and are 25 optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites

within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantify RNAi activity. Synthetic siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example Figure 11).

Example 5: Chemical Synthesis and Purification of siNA

siNA molecules can be designed to interact with various sites in the RNA message, for example target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can be synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman *et al.*, US Patent Nos. 5,804,683; 25 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe *et al.*, US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphosphoramidite groups, and 5 exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe *supra*. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman *et al.*, 10 US Patent 5,631,360, incorporated by reference herein in its entirety).

During solid phase synthesis, each nucleotide is added sequentially (3' - to 5'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator 15 are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 5'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-O-protecting group is cleaved 20 under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries 25 depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Vargeese *et al.*, USSN 10/194,875 incorporated by reference herein in its entirety or Scaringe *supra*. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that

oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30 minutes, TEA-HF is added and the reaction maintained at about 65°C for an additional 15 minutes.

Example 6: RNAi *in vitro* assay to assess siNA activity

An *in vitro* assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting cyclin D1 RNA targets. The assay comprises the system described by 10 Tuschl *et al.*, 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.*, 2000, *Cell*, 101, 25-33 adapted for use with cyclin D1 target RNA. A Drosophila extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate cyclin D1 expressing plasmid using T7 RNA 15 polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min. at 90°C followed by 1 hour at 37°C, then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be 20 monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The Drosophila lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 25 10 mM creatine phosphate, 10 ug.ml creatine phosphokinase, 100 um GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C for 10 minutes before adding RNA, then incubated at 25° C for an additional 60 minutes. Reactions are quenched with 4

volumes of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

Alternately, internally-labeled target RNA for the assay is prepared by *in vitro* transcription in the presence of [alpha-<sup>32</sup>P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-<sup>32</sup>P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by 10 Phosphor Imager<sup>®</sup> quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

In one embodiment, this assay is used to determine target sites the cyclin D1 RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the cyclin D1 RNA target, for example by analyzing the 15 assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art

Example 7: Nucleic acid inhibition of cyclin D1 target RNA *in vivo*

siNA molecules targeted to the human cyclin D1 RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity *in vivo*, for 20 example, using the following procedure. The target sequences and the nucleotide location within the cyclin D1 RNA are given in Table II and III.

Two formats are used to test the efficacy of siNAs targeting cyclin D1. First, the reagents are tested in cell culture using, for example, HELA, parathyroid adenoma, or A549 cells, to determine the extent of RNA and protein inhibition. siNA reagents (e.g.; see Tables 25 II and III) are selected against the cyclin D1 target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, HELA, parathyroid adenoma, or A549 cells. Relative amounts of target RNA are measured

versus actin using real-time PCR monitoring of amplification (e.g., ABI 7700 Taqman®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

#### Delivery of siNA to Cells

Cells (e.g., A549, parathyroid adenoma, or HEA) are seeded, for example, at  $1 \times 10^5$  10 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20nM) and cationic lipid (e.g., final concentration 2 $\mu$ g/ml) are complexed in EGM basal media (Biowhittaker) at 37°C for 30 mins in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at  $1 \times 10^3$  15 in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

#### Taqman and Lightcycler quantification of mRNA

20 Total RNA is prepared from cells following siNA delivery, for example using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For Taqman analysis, dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector 25 using 50  $\mu$ l reactions consisting of 10  $\mu$ l total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1X TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl<sub>2</sub>, 300  $\mu$ M each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AmpliTaq Gold (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase

(Promega). The thermal cycling conditions can consist of 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to  $\beta$ -actin or GAPDH mRNA in parallel TaqMan reactions.

5 For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcycler. A standard curve is generated for each primer pair using control cRNA. All values are represented as relative expression to GAPDH in each sample.

10 Western blotting

Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research*, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by 15 centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. Following washes, the 20 secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 8: Models useful to evaluate the down-regulation of cyclin D1 gene expression

*Cell Culture*

There are numerous cell culture systems that can be used to analyze reduction of cyclin 25 D1 levels either directly or indirectly by measuring downstream effects. For example, HELA, parathyroid adenoma, or A549 cells can be used in cell culture experiments to assess the efficacy of nucleic acid molecules of the invention. As such, HE LA, parathyroid adenoma, or A549 cells treated with nucleic acid molecules of the invention (e.g., siNA) targeting

cyclin D1 RNA would be expected to have decreased cyclin D1 expression capacity compared to matched control nucleic acid molecules having a scrambled or inactive sequence. In a non-limiting example, HEA, parathyroid adenoma, or A549 cells are cultured and cyclin D1 expression is quantified, for example by time-resolved immuno 5 fluorometric assay. cyclin D1 messenger-RNA expression is quantitated with RT-PCR in cultured HEA, parathyroid adenoma, or A549 cells. Untreated cells are compared to cells treated with siNA molecules transfected with a suitable reagent, for example a cationic lipid such as lipofectamine, and cyclin D1 protein and RNA levels are quantitated. Dose response assays are then performed to establish dose dependent inhibition of cyclin D1 expression.

10 In several cell culture systems, cationic lipids have been shown to enhance the bioavailability of oligonucleotides to cells in culture (Bennet, *et al.*, 1992, *Mol. Pharmacology*, 41, 1023-1033). In one embodiment, siNA molecules of the invention are 15 complexed with cationic lipids for cell culture experiments. siNA and cationic lipid mixtures are prepared in serum-free DMEM immediately prior to addition to the cells. DMEM plus additives are warmed to room temperature (about 20-25°C) and cationic lipid is added to the final desired concentration and the solution is vortexed briefly. siNA molecules are added to the final desired concentration and the solution is again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex is serially diluted into DMEM following the 10 minute incubation.

20 *Animal Models*

Evaluating the efficacy of anti-cyclin D1 agents in animal models is an important prerequisite to human clinical trials. Lead anti-cyclin D1 siNA molecules chosen from *in vitro* assays can be further tested in the following mouse model. Wang *et al.*, 1994, *Nature* 369: 669-671, produced transgenic mice containing the MMTV-LTR (mouse mammary 25 tumor virus long terminal repeat) as a promoter sequence upstream of cyclin D1. Overexpression of the transgene resulted in abnormal proliferation of mammary cells and the development of mammary adenocarcinomas. The authors proposed that reports of amplification of DNA from 11q13, the location of human cyclin D1, in 15-20% of breast cancers, may be accounted for by this gene. Treatment of these mice with siNA molecules

of the invention is expected to result in a decrease in cyclin D1 expression and a corresponding decrease in proliferation of mammary cells and decreased development of adenocarcinoma.

Example 9: RNAi mediated inhibition of cyclin D1 RNA expression

5 siNA constructs (Table III) were tested for efficacy in reducing cyclin D1 RNA expression in A549 cells. A549 cells were plated approximately 24h before transfection in 96-well plates at 5,000-7,500 cells/well, 100  $\mu$ l/well, such that at the time of transfection cells are 70-90% confluent. For transfection, annealed siNAs were mixed with the transfection reagent (Lipofectamine 2000, Invitrogen) in a volume of 50  $\mu$ l/well and 10 incubated for 20 min. at room temperature. The siNA transfection mixtures were added to cells to give a final siNA concentration of 25 nM in a volume of 150  $\mu$ l. Each siNA transfection mixture was added to 3 wells for triplicate siNA treatments. Cells were incubated at 37° for 24h in the continued presence of the siNA transfection mixture. At 24h, 15 RNA was prepared from each well of treated cells. The supernatants with the transfection mixtures were first removed and discarded, then the cells were lysed and RNA prepared from each well. Target gene expression following treatment was evaluated by RT-PCR for the target gene and for a control gene (36B4, an RNA polymerase subunit) for normalization. The triplicate data were averaged and the standard deviations determined for each treatment. 20 Normalized data were graphed and the percent reduction of target mRNA by active siNAs in comparison to their respective inverted control siNAs was determined.

Results of this study are shown in Figure 12. A siNA construct comprising 25 ribonucleotides and 3'-terminal dithymidine caps (RPI#30988/31064) was compared to a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31300/3130), which was also compared to a matched chemistry inverted control (RPI#31312/31313). In addition, the siNA constructs were also compared to untreated cells, cells transfected with lipid and scrambled siNA constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control).

As shown in the figure, both siNA constructs show significant reduction of cyclin D1 RNA expression..

Example 10: Indications

Particular conditions and disease states that can be associated with cyclin D1 expression modulation include but are not limited oncology and proliferation related indications and conditions, including breast cancer, cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, multiple myeloma, ovarian cancer, melanoma, colorectal cancer, prostate cancer, lung cancer, bladder cancer, esophageal cancer, glioblastoma, and proliferative conditions such as restinosis, and any other indications that can respond to the level of cyclin D1 in a cell or tissue, alone or in combination with other therapies.

The use of radiation treatments and chemotherapeutics such as Gemcytabine and cyclophosphamide are non-limiting examples of chemotherapeutic agents that can be combined with or used in conjunction with the nucleic acid molecules (e.g. siNA molecules) of the instant invention. Those skilled in the art will recognize that other anti-cancer compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention (e.g. siNA molecules) and are hence within the scope of the instant invention. Such compounds and therapies are well known in the art (see for example *Cancer: Principles and Practice of Oncology*, Volumes 1 and 2, eds Devita, V.T., Hellman, S., and Rosenberg, S.A., J.B. Lippincott Company, Philadelphia, USA; incorporated herein by reference) and include, without limitations, folates, antifolates, pyrimidine analogs, fluoropyrimidines, purine analogs, adenosine analogs, topoisomerase I inhibitors, anthrapyrazoles, retinoids, antibiotics, anthacyclins, platinum analogs, alkylating agents, nitrosoureas, plant derived compounds such as vinca alkaloids, epipodophyllotoxins, tyrosine kinase inhibitors, taxols, radiation therapy, surgery, nutritional supplements, gene therapy, radiotherapy, for example 3D-CRT, immunotoxin therapy, for example ricin, and monoclonal antibodies. Specific examples of chemotherapeutic compounds that can be combined with or used in conjunction with the nucleic acid molecules of the invention

include, but are not limited to, Paclitaxel; Docetaxel; Methotrexate; Doxorubicin; Edatrexate; Vinorelbine; Tomaxifen; Leucovorin; 5-fluoro uridine (5-FU); I onotecan; Cisplatin; Carboplatin; Amsacrine; Cytarabine; Bleomycin; Mitomycin C; Dactinomycin; Mithramycin; Hexamethylmelamine; Dacarbazine; L-asperginate; Nitrogen mustard; 5 Melphalan, Chlorambucil; Busulfan; Ifosfamide; 4-hydroperoxycyclophosphamide, Thiotepa; Irinotecan (CAMPTOSAR®, CPT-11, Camptothecin-11, Campto) Tamoxifen, Herceptin; IMC C225; ABX-EGF; and combinations thereof are non-limiting examples of compounds and/or methods that can be combined with or used in conjunction with the nucleic acid molecules (e.g. siNA) of the instant invention. Those skilled in the art will 10 recognize that other drug compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention (e.g., siNA molecules) are hence within the scope of the instant invention.

Example 11: Diagnostic uses

The siNA molecules of the invention can be used in a variety of diagnostic applications, 15 such as in identifying molecular targets such as RNA in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells 20 or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and 25 function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies

(e.g., multiple siNA molecules targeted to different genes, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siNA molecules of this invention are well known in the art, and include detection of the presence 5 of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example fluorescence resonance emission transfer (FRET).

In a specific example, siNA molecules that can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that can cleave 10 only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that can cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the 15 "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis requires two siNA molecules, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage 20 products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA 25 whose protein product is implicated in the development of the phenotype (*i.e.*, disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios is correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this

disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as 5 those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

10 It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described 15 herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested 20 without undue experimentation toward identifying siNA molecules with improved RNAi activity.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting 25 essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions 'hereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it

should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the 5 description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

**Table I: cyclin D1 Accession Numbers**

NM_003914	Homo sapiens cyclin A1 (CCNA1), mRNA
NM_001237	Homo sapiens cyclin A2 (CCNA2), mRNA
NM_031966	Homo sapiens cyclin B1 (CCNB1), mRNA
NM_004701	Homo sapiens cyclin B2 (CCNB2), mRNA
NM_033670	Homo sapiens cyclin B3 (CCNB3), transcript variant 1, mRNA
NM_033671	Homo sapiens cyclin B3 (CCNB3), transcript variant 2, mRNA
NM_033031	Homo sapiens cyclin B3 (CCNB3), transcript variant 3, mRNA
NM_005190	Homo sapiens cyclin C (CCNC), mRNA
NM_053056	Homo sapiens cyclin D1 (PRAD1): parathyroid adenomatosis 1) (CCND1), mRNA
NM_001758	Homo sapiens cyclin D1 (PRAD1): parathyroid adenomatosis 1) (CCND1), mRNA
NM_001759	Homo sapiens cyclin D2 (CCND2), mRNA
NM_001760	Homo sapiens cyclin D3 (CCND3), mRNA
NM_001238	Homo sapiens cyclin E1 (CCNE1), transcript variant 1, mRNA
NM_057182	Homo sapiens cyclin E1 (CCNE1), transcript variant 2, mRNA
NM_057749	Homo sapiens cyclin E2 (CCNE2), transcript variant 1, mRNA
NM_057735	Homo sapiens cyclin E2 (CCNE2), transcript variant 2, mRNA
NM_004702	Homo sapiens cyclin E2 (CCNE2), transcript variant 3, mRNA
NM_016323	Homo sapiens cyclin E binding protein 1 (LOC51191), mRNA
NM_001761	Homo sapiens cyclin F (CCNF), mRNA
NM_004060	Homo sapiens cyclin G1 (CCNG1), mRNA
NM_004354	Homo sapiens cyclin G2 (CCNG2), mRNA
NM_005255	Homo sapiens cyclin G associated kinase (GAK), mRNA
NM_001239	Homo sapiens cyclin H (CCNH), mRNA
NM_006835	mRNA
	Homo sapiens cyclin L ania-6a (LOC57018), mRNA
NM_020307	mRNA
NM_020348	Homo sapiens cyclin M1 (CCNM1), mRNA

NM_017649	Homo sapiens cyclin M2 (CNNM2), 1..RNA
NM_017623	Homo sapiens cyclin M3 (CNNM3), mRNA
NM_001240	Homo sapiens cyclin T1 (CCNT1), mRNA
NM_001241	Homo sapiens cyclin T2 (CCNT2), transcript variant a, mRNA
NM_058241	Homo sapiens cyclin T2 (CCNT2), transcript variant b, mRNA

#### Cyclin-dependent Kinases

NM_033379	Homo sapiens cell division cycle 2, G1 to S and G2 to M (CDC2), transcript variant 2, mRNA
NM_001786	Homo sapiens cell division cycle 2, G1 to S and G2 to M (CDC2), transcript variant 1, mRNA
NM_001798	Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA
NM_052827	Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 2, mRNA
NM_016550	Homo sapiens HeLa cyclin-dependent kinase 2 interacting protein (CDNP), mRNA
NM_001258	Homo sapiens cyclin-dependent kinase 3 (CDK3), mRNA
NM_000075	Homo sapiens cyclin-dependent kinase 4 (CDK4), transcript variant 1, mRNA
NM_052984	Homo sapiens cyclin-dependent kinase 4 (CDK4), transcript variant 2, mRNA
NM_004935	Homo sapiens cyclin-dependent kinase 5 (CDK5), mRNA
NM_003885	Homo sapiens cyclin dependent kinase 5, regulatory subunit 1 (p35) (CDK5R1), mRNA
NM_003936	Homo sapiens cyclin dependent kinase 5, regulatory subunit 2 (p39) (CDK5R2), mRNA
NM_001259	Homo sapiens cyclin-dependent kinase 6 (CDK6), mRNA
NM_001799	Homo sapiens cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase) (CDK7), mRNA
NM_001260	Homo sapiens cyclin-dependent kinase 8 (CDK8), mRNA
NM_001261	Homo sapiens cyclin-dependent kinase 9 (CDK9), mRNA
NM_003674	mRNA
NM_052987	Homo sapiens cyclin-dependent kinase (CDC2-related kinase) 10 (CDK10), transcript variant 2, mRNA
NM_052988	Homo sapiens cyclin-dependent kinase (CDC2-like) 10 (CDK10), transcript variant 3, mRNA

Table II: cyclin D1 siRNA and Target Sequences

NM_053056 (LCCND1)		Seq ID	UpPos	Upper seq	Seq ID	LPos	Lower seq	Seq ID
Pos	Seq							
3	CACGGACUACAGGGAGUU	1	3	CACGGACUACAGGGAGUU	1	21	AACUCCCCCGUAGUCCGUG	240
21	UUUGUUGAAGUUGCAAGU	2	21	UUUGUUGAAGUUGCAAGU	2	39	ACUUUUGCAACUUCACAAA	241
39	UCUGGAGGCCUCCAGAGGG	3	39	UCUGGAGGCCUCCAGAGGG	3	57	CCUCUGGGGUCCUAGGGA	242
57	GCUGUGGGCAGUAGCAG	4	57	GCUGUGGGCAGUAGCAG	4	75	CUGGUACUGGCCGGAOAGC	243
75	GCGAGCAGCAGAGUCCGCA	5	75	GCGAGCAGCAGAGUCCGCA	5	93	UGCGGACUCUGCUCUCGCG	244
93	ACGCUCGGCGAGGGCAG	6	93	ACGCUCGGCGAGGGCAG	6	111	CUGCCCCUCCGGGAGCGU	245
111	GAAGAGCGCGAGGGAGCC	7	111	GAAGAGCGCGAGGGAGCC	7	129	GCGCUCCUCUCCGGCUUC	246
129	CGGGGAGCAGAAGCGAGA	8	129	CGGGGAGCAGAAGCGAGA	8	147	UCUCGGCUUCUGCGGCCG	247
147	AGCCGAGGCCGGACCCAGC	9	147	AGCCGAGGCCGGACCCAGC	9	165	GCUGGGGUUCGCGGCCGCU	248
165	CCAGGAACCACAGCCCUCC	10	165	CCAGGAACCACAGCCCUCC	10	183	GAAGGGGUUCGGGUCCUGGG	249
183	CCAGGOUUGCCAGGAAGAG	11	183	CCAGGOUUGCCAGGAAGAG	11	201	CUUCUCCUGGGCGUCGGGG	250
201	GCCCCAGCCAUGGAACACC	12	201	GCCCCAGCCAUGGAACACC	12	219	GGGUUCCAUAGGGCGGGC	251
219	CAGCUCCUGUGCGUGGAAG	13	219	CAGCUCCUGUGCGUGGAAG	13	237	CUUCGCAAGCACAGGAGCUG	252
237	GUGGAACCAUCCGCCGCC	14	237	GUGGAACCAUCCGCCGCC	14	255	CGGGGGGGGAUGGUUUUCCAC	253
255	GCGUACCCGAUGCAACC	15	255	GCGUACCCGAUGCAACC	15	273	GGUUGGGCAUCGGGUACGC	254
273	CUCCUCAACGACCGGGUGC	16	273	CUCCUCAACGACCGGGUGC	16	291	GCACCCGGUUCGUAGGGAG	255
291	CUGCCGGCCAUGUGAAGG	17	291	CUGCCGGCCAUGUGAAGG	17	309	CCUUCAGCAUGGGCCGAG	256
309	GCGGAGGAGACCUGCGGC	18	309	GCGGAGGAGACCUGCGGC	18	327	GCGCAGGGGUUCUCCGGC	257
327	CCCUUCCGGGUCCUACUCA	19	327	CCCUUCCGGGUCCUACUCA	19	345	UGAAGUAGGAGACCCGAGGG	258
345	AAAUGUGUGGAGAAGGGG	20	345	AAAUGUGUGGAGAAGGGG	20	363	CCUCUUCUCCGGACACAUU	259
363	GUCCUGCCGUCCAUUGCGGA	21	363	GUCCUGCCGUCCAUUGCGGA	21	381	UCCGCAUGGGACGGAGGAC	260
381	AAGAUUCCGUCCACCUUGA	22	381	AAGAUUCCGUCCACCUUGA	22	399	UCCAGGGUUCGGGACGAUCU	261
399	AUGCUUCCGGGUCCGGAGG	23	399	AUGCUUCCGGGUCCGGAGG	23	417	CCUCGAGGACCCUCCGGCAU	262
417	GAACAGGAAGUGCGAGGG	24	417	GAACAGGAAGUGCGAGGG	24	435	CCUCUCCGGACUUCGUUC	263
435	GAGGUUUCUCCGCCUGGCA	25	435	GAGGUUUCUCCGCCUGGCA	25	453	UGGGGUUCAGGGUAGUUCAU	264
453	AUGAACUACCUCCGGCU	26	453	AUGAACUACCUCCGGACCGU	26	471	AGGGGUUCAGGGUAGUUCAU	265
471	UUCCUUCCGGGUCCGGCG	27	471	UUCCUUCCGGGUCCGGCG	27	489	CGGGCUUCAGGGGACAGGGAA	266
489	GUGAAAAAGAGCCGCCUGC	28	489	GUGAAAAAGAGCCGCCUGC	28	507	GCAGGGGCCGCCACUUC	267
507	CAGCUUCCGGGGGCCACUU	29	507	CAGCUUCCGGGGGCCACUU	29	525	AAGUGGGCCAGCAGCUC	268
525	UGCAUGUUCUCCGCCUCUA	30	525	UGCAUGUUCUCCGCCUCUA	30	543	UAGAGGGCAGAACAUCAU	269
543	AAGAUGAAGGAGCAUCC	31	543	AAGAUGAAGGAGCAUCC	31	561	GGAUUGUUCUCCUCAUCUU	270

561	CCCCUGACGGCCGAGAAGC	32	561	CCCCUGACGGCCGAGAAGC	32	579	GUUUCUGGCCGAGAAC	32	597	GUUUCUGGCCGAGAAC	271
579	CUGUGCAUCUACACCGACA	33	579	CUGUGCAUCUACACCGACA	33	615	CUCGGGGCCGGGAUGAGUU	272	615	CUCGGGGCCGGGAUGAGUU	272
597	AACUCCAUCCGGCCGAGG	34	597	AACUCCAUCCGGCCGAGG	34	633	GCUCCAUUGCAGCACUC	273	633	GCUCCAUUGCAGCACUC	273
615	GAGCUGCUGCAAAGGAGC	35	615	GAGCUGCUGCAAAGGAGC	35	651	GCUCGUUACCCAGGAGCAG	274	651	GCUCGUUACCCAGGAGCAG	274
633	CUGCUCCUGGUGAACAAAGC	36	633	CUGCUCCUGGUGAACAAAGC	36	669	CGGCCAGGUUCCACUUGAG	275	669	CGGCCAGGUUCCACUUGAG	275
651	CUCAAGUGGAACCCGGCG	37	651	CUCAAGUGGAACCCGGCG	37	687	AUCUGUGGGGUCAUUGC	276	687	AUCUGUGGGGUCAUUGC	276
669	GCAAUGACCCGCAACGAUU	38	669	GCAAUGACCCGCAACGAUU	38	705	AGAGGAAGGUCAUUGAA	277	705	AGAGGAAGGUCAUUGAA	277
687	UUCAUUGAACACUUCUICU	39	687	UUCAUUGAACACUUCUICU	39	723	CCGCCUCUGGGCAUUUGGA	278	723	CCGCCUCUGGGCAUUUGGA	278
705	UCCAAAUGCCAGAGGGGG	40	705	UCCAAAUGCCAGAGGGGG	40	741	UGAUUCGUUUGUUCUCCUC	280	741	UGAUUCGUUUGUUCUCCUC	280
723	GAGGAGAACAAACAGAUCA	41	723	GAGGAGAACAAACAGAUCA	41	759	UCUGGCCUGGUUUGGGAU	281	759	UCUGGCCUGGUUUGGGAU	281
741	AUCCGCAAAACACGGCGAGA	42	741	AUCCGCAAAACACGGCGAGA	42	777	CACAGAGGCCAACGAAGGU	282	777	CACAGAGGCCAACGAAGGU	282
759	ACCUUCGUUGCCCCUJUGU	43	759	ACCUUCGUUGCCCCUJUGU	43	795	UGAACUUCACACUUGGGC	283	795	UGAACUUCACACUUGGGC	283
777	GCCACAGAUGUGAACUCA	44	777	GCCACAGAUGUGAACUCA	44	813	UGGAGGGGGAUUUGGAAAU	284	813	UGGAGGGGGAUUUGGAAAU	284
795	AUUCCCAUCGGCCCUCCA	45	795	AUUCCCAUCGGCCCUCCA	45	831	CGCUCCCCGGCUGCCACCAU	285	831	CGCUCCCCGGCUGCCACCAU	285
813	AUGGUGGCAGGGGGAGCG	46	813	AUGGUGGCAGGGGGAGCG	46	849	CUUGCACUGGGGGCCACCCAC	286	849	CUUGCACUGGGGGCCACCCAC	286
831	GUUGGUCCCCGAGUGGAAG	47	831	GUUGGUCCCCGAGUGGAAG	47	867	GGCUCCUCAGGUUUGGGCC	287	867	GGCUCCUCAGGUUUGGGCC	287
849	GGCUUGAACCCUGAGGCC	48	849	GGCUUGAACCCUGAGGCC	48	885	AGGACAGGAAGUUGUUGGG	288	885	AGGACAGGAAGUUGUUGGG	288
867	CCCAACAAACUUCUCCGUCCU	49	867	CCCAACAAACUUCUCCGUCCU	49	903	AGCGUGUGAGGGGGUAGUA	289	903	AGCGUGUGAGGGGGUAGUA	289
885	UACUACCGCCUCACACGGU	50	885	UACUACCGCCUCACACGGU	50	921	UGAUACUCUUGGGAGGGAA	290	921	UGAUACUCUUGGGAGGGAA	290
903	UUCUCCUCUCCAGAGUGAUCA	51	903	UUCUCCUCUCCAGAGUGAUCA	51	939	GGCGUICUGGGUCACACUU	291	939	GGCGUICUGGGUCACACUU	291
921	AAGUGUACCCAGACUGCC	52	921	AAGUGUACCCAGACUGCC	52	957	GCUCUCGGCCGGGGAG	292	957	GCUCUCGGCCGGGGAG	292
939	CUCCGGGCCUGCCAGGAGC	53	939	CUCCGGGCCUGCCAGGAGC	53	975	CCAGCAGGGGUUCUGAUCUG	293	975	CCAGCAGGGGUUCUGAUCUG	293
957	CAGAUCGAAGCCCCUGGU	54	957	CAGAUCGAAGCCCCUGGU	54	993	CCUGGGCAGGGGUUGACUC	294	993	CCUGGGCAGGGGUUGACUC	294
975	GAGUCAAGGCCUGGCCAGG	55	975	GAGUCAAGGCCUGGCCAGG	55	1011	GGUCCAUUGUUCGGGGGG	295	1011	GGUCCAUUGUUCGGGGGG	295
993	GCCCCAGCAAAACUUGGACC	56	993	GCCCCAGCAAAACUUGGACC	56	1029	CCUCCUCUCCUCUCCUC	296	1029	CCUCCUCUCCUCUCCUC	296
1011	CCCAAGGGCCGGAGGAGG	57	1011	CCCAAGGGCCGGAGGAGG	57	1047	CCUCCUCUCCUCUCCUC	297	1047	CCUCCUCUCCUCUCCUC	297
1029	GAGGAAGGGAGGGAGGG	58	1029	GAGGAAGGGAGGGAGGG	58	1065	UGCAAGCAGGGGUACCCUC	298	1065	UGCAAGCAGGGGUACCCUC	298
1047	GAGGUGGACCCUGGUUGCA	59	1047	GAGGUGGACCCUGGUUGCA	59	1119	CCGGCAGGUCCGGGGGG	299	1119	CCGGCAGGUCCGGGGGG	299
1065	ACACCCACCCGACGUCCGG	60	1065	ACACCCACCCGACGUCCGG	60	1101	GCCCCUCAUGUCCACGUC	300	1101	GCCCCUCAUGUCCACGUC	300
1083	GACGGUGGACACUUGAGGGC	61	1083	GACGGUGGACACUUGAGGGC	61	1119	GGGGCCGGCCUGGGGGCG	301	1119	GGGGCCGGCCUGGGGGCG	301
1101	CGCCCAAGCCAGGGGGGCC	62	1101	CGCCCAAGCCAGGGGGGCC	62	1137	CUGCGUGGGGGGGGGGG	302	1137	CUGCGUGGGGGGGGGGG	302
1119	CACCCGCCACCCGACGGAG	63	1119	CACCCGCCACCCGACGGAG	63	1155	CCUGGGGGCCUGCCGCC	303	1155	CCUGGGGGCCUGCCGCC	303
1137	GGGCGAGGGGGCCAGCAG	64	1137	GGGCGAGGGGGCCAGCAG	64	1173	GGGACUGUAGGGGGAGC	304	1173	GGGACUGUAGGGGGAGC	304
1155	GUGGUCCCCUAGACGUCC	65	1155	GUGGUCCCCUAGACGUCC	65	1191	AAAUGCUCCGGAGAGGG	305	1191	AAAUGCUCCGGAGAGGG	305
1173	CUCCUCUCCGGAGCAUUU	66	1173	CUCCUCUCCGGAGCAUUU	66	1209	GUUUUCCUUCUGGUUAUC	306	1209	GUUUUCCUUCUGGUUAUC	306
1191	UGAUACCAGAAGGGAAAAGC	67	1191	UGAUACCAGAAGGGAAAAGC	67						

1209	CUUCAUCUCCUUGUUGUU	68	1209	CUUCAUUCUCUUGUUGUU	68	1227	ACAAACAAGGAGAAUUGAAG	307
1227	UGGUUGUUUUYGCUUUGC	69	1227	UGGUUGUUUUYGCUUUGC	69	1245	GCAAAGGAAAAAACACCA	308
1245	CUCUUCUCCCUUCCAUUCU	70	1245	CUCUUCUCCCUUCCAUUCU	70	1263	AGAUGGAAGGGGGAAAGAG	309
1263	UCUGACUUAGCAAAAGAA	71	1263	UCUGACUUAGCAAAAGAA	71	1281	UUCUUUUGCUUAAGUCAGA	310
1281	AAAAGAUUACCCAAACU	72	1281	AAAAGAUUACCCAAACU	72	1299	AGUUUUUUGGUAAUUCUUUU	311
1299	UGCUUUAAGAGAGAGA	73	1299	UGCUUUAAGAGAGAGA	73	1317	UCUCUCUUCUUUAAGACAA	312
1317	AGAGAAAAAAAUAGUA	74	1317	AGAGAAAAAAAUAGUA	74	1335	UACUAUUUUUUUUCUUCU	313
1335	AUUGCAUACCCUGAGCG	75	1335	AUUGCAUACCCUGAGCG	75	1353	CGCUCAGGCUAUGCAAAAU	314
1353	GGUGGGGAGGGGUUGU	76	1353	GGUGGGGAGGGGUUGU	76	1371	ACAAACCCUCCUCCCCCACC	315
1371	UGCUACAGAUGAUAGAGGA	77	1371	UGCUACAGAUGAUAGAGGA	77	1389	UCCUCUACAUCAUCUGUAGCA	316
1389	AUUIUAUACCCCAUAUAC	78	1389	AUUIUAUACCCCAUAUAC	78	1407	GAUUAUUGGGGUAAUAAAUAU	317
1407	CAACUCGUUUUAUUAUAA	79	1407	CAACUCGUUUUAUUAUAA	79	1425	UAAAUAUAAAACGAGUUG	318
1425	AUGUACUUGUUUCUCUGUU	80	1425	AUGUACUUGUUUCUCUGUU	80	1443	AACAGAAACAUAGUACAU	319
1443	UGUAAGAAUAGGCAUUAAC	81	1443	UGUAAGAAUAGGCAUUAAC	81	1461	GUUAUGGCCAUUCUUAUCA	320
1461	CACAAAGGAGGGCGUCUCGG	82	1461	CACAAAGGAGGGCGUCUCGG	82	1479	CCGAGACGCCUUUJUGUG	321
1479	GGAGAGGUUAGGUUCCAU	83	1479	GGAGAGGUUAGGUUCCAU	83	1497	AUGGAACCUAUCCUCUCC	322
1497	UCCUUUACGUGUUAAAAAA	84	1497	UCCUUUACGUGUUAAAAAA	84	1515	UUUUAAAACGUGUUAGGA	323
1515	AAAAGCAUAAAACAUUUU	85	1515	AAAAGCAUAAAACAUUUU	85	1533	AAAAGUUUUUUAUGCUUUUU	324
1533	UAAAACAUAGAAAAAUUC	86	1533	UAAAACAUAGAAAAAUUC	86	1551	GAUUUUUUCUAUGUUUUUA	325
1551	CAGCAAACCAUUUUAAAAG	87	1551	CAGCAAACCAUUUUAAAAG	87	1569	CUUUUAAAUGGUUUGUCUG	326
1569	GUAGAAGGGUUUAGGU	88	1569	GUAGAAGGGUUUAGGU	88	1587	ACCUAAAACCCUUCUUCUAC	327
1587	UAGAAAAACAUAUUCUUGU	89	1587	UAGAAAAACAUAUUCUUGU	89	1605	ACAAAGAAUUAUGUUUUUCUA	328
1605	UGCUUUUCUGAUAAAAGCA	90	1605	UGCUUUUCUGAUAAAAGCA	90	1623	UGCUUUUAUCAGGAAAAGCA	329
1623	ACAGCUGUAGUGGGUUUCU	91	1623	ACAGCUGUAGUGGGUUUCU	91	1641	AGAACCCACAUACGCGU	330
1641	UAGGCAUCUCUGUACUJUG	92	1641	UAGGCAUCUCUGUACUJUG	92	1659	CAAAGUACAGAGAUGGCCUA	331
1659	GCUUUCACCUUUAUCUGGC	93	1659	GCUUUCACCUUUAUCUGGC	93	1677	UACAUUGCAUAGGAAAGC	332
1677	AGUCACUUUAAGCUAU	94	1677	AGUCACUUUAAGCUAU	94	1695	AAUGACUUAUAAAGUGACU	333
1695	UGUAUGUUUAUUAUUCG	95	1695	UGUAUGUUUAUUAUUCG	95	1713	CGGAAUUAUAAAACAUACAU	334
1713	GUAGGUAGAUGGUAAACCU	96	1713	GUAGGUAGAUGGUAAACCU	96	1731	AGGUUACACAUACUACUAC	335
1731	UCUUUCACCUUUAUCUGGC	97	1731	UCUUUCACCUUUAUCUGGC	97	1749	GCCAUUGAAUAGGUAGAAGA	336
1749	CUGAAGUCACCUUJUGGU	98	1749	CUGAAGUCACCUUJUGGU	98	1767	AACCAAGAGGGUGACUUCAG	337
1767	UACAGUAGCGUAGCGUGGC	99	1767	UACAGUAGCGUAGCGUGGC	99	1785	GCCACCGCUACGCCUACUGUA	338
1785	CCGUGUGCAUGGUUJUGC	100	1785	CCGUGUGCAUGGUUJUGC	100	1803	GCAAAGGACAUGCACACGG	339
1803	CGCCUGUGACCCACCCCC	101	1803	CGCCUGUGACCCACCCCC	101	1821	GGGGUGGGUGGUACAGGG	340
1821	CAACAAACCAUCCAGUGAC	102	1821	CAACAAACCAUCCAGUGAC	102	1839	GUACUGUGAUGGUUJUGU	341
1839	CAAACCAUCCAGUGGAGGU	103	1839	CAAACCAUCCAGUGGAGGU	103	1857	ACUCCACUCCAGUGGUUUG	342

1857	UUUGUCGGCACAGCCAG	104	1857	UUUGUCGGCACAGCCAG	104	1875	CUGGGUGGGGGGGGACAAAA	343
1875	GCGUAGCAGGGGGAAA	105	1875	GCGUAGCAGGGGGAAA	105	1893	UUUCCCGACCCUGCUACGCC	344
1893	AGGCCACCUCCGUCCACUCC	106	1893	AGGCCACCUCCGUCCACUCC	106	1911	GGAGUGGGCACAGGGGCCU	345
1911	CUACGAUACGGCUACAUAA	107	1911	CUACGAUACGGCUACAUAA	107	1929	UUAUAGUAGCGUAUCGUAG	346
1929	AAGAGAAGACGAAAUAUGUG	108	1929	AAGAGAAGACGAAAUAUGUG	108	1947	CACAUUUUUCGUUCUUCUUCU	347
1947	GACAUAAUAAUUCUAAUUU	109	1947	GACAUAAUAAUUCUAAUUU	109	1965	AAAUAAGAAUAAUUAUAGUC	348
1965	UUUAUACUCUUCUAAUUU	110	1965	UUUAUACUCUUCUAAUUU	110	1983	AAAUAAGGAAGAGUAAAAA	349
1983	UUUGUAGUGACCUGUUAUG	111	1983	UUUGUAGUGACCUGUUAUG	111	2001	CAUAAACAGGGACUACUCAA	350
2001	GAGAUGCUGGUUUCUACCC	112	2001	GAGAUGCUGGUUUCUACCC	112	2019	GGUAGAAAACCAGCAUCUC	351
2019	CCAACCGGCCUGCCAG	113	2019	CCAACCGGCCUGCCAG	113	2037	CUGGCUGCAGGGCCGUUGGG	352
2037	GCUUCACGUCCAGGUUCAAC	114	2037	GCUUCACGUCCAGGUUCAAC	114	2055	GUUGAACCUUGGACGUUGAGC	353
2055	CCCACAGCUACUUGGUUUG	115	2055	CCCACAGCUACUUGGUUUG	115	2073	AAAACCAAGGUAGGUUGGG	354
2073	GUGUUCUUCUCAUAAUCU	116	2073	GUGUUCUUCUCAUAAUCU	116	2091	AGGAAUAGGAAGAAACAC	355
2091	UAAAACCAUUCUCAUUCCA	117	2091	UAAAACCAUUCUCAUUCCA	117	2109	UIGGAAAAGGAAAGGUUUUA	356
2109	AAGCACUUUCAGUCCAAUA	118	2109	AAGCACUUUCAGUCCAAUA	118	2127	UAUUGGGACUAGAAAGGUUU	357
2127	AGGUGUAGGAAAUAGGCCU	119	2127	AGGUGUAGGAAAUAGGCCU	119	2145	AGCGCUAUUUCUACACCU	358
2145	UGUUUUUGUUGUGUGCA	120	2145	UGUUUUUGUUGUGUGCA	120	2163	UGCACACACAAACAAAAACA	359
2163	AGGGAGGGCAGUUUCUAA	121	2163	AGGGAGGGCAGUUUCUAA	121	2181	UJAGAAAACUGCCCCUCCU	360
2181	AUGGAAUGGUUUGGGAAUA	122	2181	AUGGAAUGGUUUGGGAAUA	122	2199	UAUUCCCAAAACCUUCCAU	361
2199	AUCCAUQUACUUGUUGCA	123	2199	AUCCAUQUACUUGUUGCA	123	2217	UGCAAACAAAGUACAUUGGAU	362
2217	AAGCAGGACUUUGAGGCAA	124	2217	AAGCAGGACUUUGAGGCAA	124	2235	UJGCCUCAAGUCCUGCUU	363
2235	AGUGUGGGCACUGGGUG	125	2235	AGUGUGGGCACUGGGUG	125	2253	CACCCACUGCCCCACACU	364
2253	GGCAGUGGGAGGGGGGGU	126	2253	GGCAGUGGGAGGGGGGGU	126	2271	ACACCCACUCCACUGGCC	365
2271	UUUGGGAGGGUGGGGCCA	127	2271	UUUGGGAGGGUGGGGCCA	127	2289	UGGCACGGAGCCUCCAAA	366
2289	AGUCAGAAGAAAAGGUU	128	2289	AGUCAGAAGAAAAGGUU	128	2307	AACCUUUUUCUUCUUGACU	367
2307	UGGCAUUCUCACAUUGCCA	129	2307	UGGCAUUCUCACAUUGCCA	129	2325	UGGCCAAUGGAGGUUGCAA	368
2325	AGGAUGAUAAAGGUUCUUC	130	2325	AGGAUGAUAAAGGUUCUUC	130	2343	GAAAAGAACUUAUCUCCU	369
2343	CCUUUUUCUAAAAGAAGUU	131	2343	CCUUUUUCUAAAAGAAGUU	131	2361	AACUUUCUAAAAGAAAAGG	370
2361	UGAAGUUAGGAUUCUUU	132	2361	UGAAGUUAGGAUUCUUU	132	2379	AAGGAUUCUAAAAGUACU	371
2379	UGGUGGCCAACUGGUUG	133	2379	UGGUGGCCAACUGGUUG	133	2397	CAAACACCAAGUUGGCCACCA	372
2397	GAAAAGUAGGGGACCUAGAG	134	2397	GAAAAGUAGGGGACCUAGAG	134	2415	CUCUGAGGUCCCUACUUUC	373
2415	GUUUUACCUAGGAACAGG	135	2415	GUUUUACCUAGGAACAGG	135	2433	CCUGUUCUUGGUAAAACCAC	374
2433	GUGGUUUUAAGGUUAUC	136	2433	GUGGUUUUAAGGUUAUC	136	2451	GAUAAACCCUUAAAACCAC	375
2451	CUAGAUGUUUCACACGG	137	2451	CUAGAUGUUUCACACGG	137	2469	CCGGUGUGGAAAACAUUAAG	376
2469	GAAGGUUUUAACACCUAA	138	2469	GAAGGUUUUAACACCUAA	138	2487	UAGUGUUAAAACCUUC	377
2487	AAAUAUUAUAAAUAUAGUU	139	2487	AAAUAUUAUAAAUAUAGUU	139	2505	ACUUAUAAAUAUAAAUAU	378

2505	UAGGCUAAAAGUUAUU	140	2505	UAGGCUAAAAGUUAUU	140	2523	AUAUACUUUUAGCCUUA	379
2523	UUAUUGAGAGGAUGUUC	141	2523	UUAUUGAGAGGAUGUUC	141	2541	UGAACAUCCUCUGCAAUAA	380
2541	AUAAGGCCAGUAUGAUUA	142	2541	AUAAGGCCAGUAUGAUUA	142	2559	UAAAUCAUACUGGCCUUAU	381
2559	AUAAAUGCACAUUCUCCUU	143	2559	AUAAAUGCACAUUCUCCUU	143	2577	AAGGGGAGAUUGCAUUAU	382
2577	UGAUUUAAACACAGAU	144	2577	UGAUUUAAACACAGAU	144	2595	UAUCUGUGUGUUUAAAUC	383
2595	ACACACACACACACACA	145	2595	ACACACACACACACACA	145	2613	UGUGUGUGUGUGUGUGU	384
2613	ACACACACAAACCUCUUC	146	2613	ACACACACAAACCUCUUC	146	2631	GCAGAAGGUUGUGUGUGU	385
2631	CCUUGAUGUUAAGAUUU	147	2631	CCUUGAUGUUAAGAUUU	147	2649	AAAUCUGUAACAUAAAAG	386
2649	UAUAUACAGUUUUUUUUA	148	2649	UAUAUACAGUUUUUUUUA	148	2667	UAAAAAAUAAACUGUAUUA	387
2667	AAGAUAGAUCCUUUUUAAG	149	2667	AAGAUAGAUCCUUUUUAAG	149	2685	CUAUAAAAGGAUCUAUCUU	388
2685	GGUGGAGAAAAAAACAAUC	150	2685	GGUGGAGAAAAAAACAAUC	150	2703	AGAUUGUUUUUUUCUACCC	389
2703	UGGAGAGAAAAAAACACAC	151	2703	UGGAGAGAAAAAAACACAC	151	2721	GUUGGGGUUUUUUCUCC	390
2721	CAAAGACAUUGAUUCAGCC	152	2721	CAAAGACAUUGAUUCAGCC	152	2739	GGCGUGAAUCAUGUCUUG	391
2739	CUGUUUGGGCUGUUCGAGA	153	2739	CUGUUUGGGCUGUUCGAGA	153	2757	UCUGGGAAAAGGCCAAACAG	392
2757	AGUCAUCUGAUUGGACAGG	154	2757	AGUCAUCUGAUUGGACAGG	154	2775	CGUGGUCCAAUCAGAUGACU	393
2775	GCAUGGGUGGCGAAGAAAAU	155	2775	GCAUGGGUGGCGAAGAAAAU	155	2793	AUUUUCCUUGCACCCAUGC	394
2793	UJAGGGUACUAAACGUAG	156	2793	UJAGGGUACUAAACGUAG	156	2811	CUAGGGUJAGUACCCUUA	395
2811	GUUCGGGUUCCGAUAAUUC	157	2811	GUUCGGGUUCCGAUAAUUC	157	2829	GAUUCUCAUJGGAAACCGAAC	396
2829	CUUAUCCCUGGCCUUC	158	2829	CUUAUCCCUGGCCUUC	158	2847	GAAGGGGGCAGGGGAUAAAG	397
2847	CUUAAAACAUUAGUGAC	159	2847	CUUAAAACAUUAGUGAC	159	2865	GUACUAAAGUUUUUUAAAG	398
2865	CAAAAUAGACAAUUGCAC	160	2865	CAAAAUAGACAAUUGCAC	160	2883	GUGCAAAUUGUCUUAUUUG	399
2883	CAUCUUGGCCUAUUAUUC	161	2883	CAUCUUGGCCUAUUAUUC	161	2901	GAAUUACAUAGCCAAAGAUG	400
2901	CUUGUAUUUUUUUAUUAGG	162	2901	CUUGUAUUUUUUUAUUAGG	162	2919	CCUUAAAACAUUACAAAG	401
2919	GAAGGUUGAAGGGAGGUG	163	2919	GAAGGUUGAAGGGAGGUG	163	2937	CACCUUCCUUAACACUUC	402
2937	GGCAAGAGUGGGAGGUG	164	2937	GGCAAGAGUGGGAGGUG	164	2955	CAGCCUCCACACUUCUIGCC	403
2955	GACGUGUGAGGGAGGACAG	165	2955	GACGUGUGAGGGAGGACAG	165	2973	CUGUCCUCCUCACACGUC	404
2973	GGGGGGAGGGAGGUGUGAGG	166	2973	GGGGGGAGGGAGGUGUGAGG	166	2991	CCUCACACCUCCUCCGCC	405
2991	GAGGAGGGCUCCGAGGGA	167	2991	GAGGAGGGCUCCGAGGGA	167	3009	UCCCCUCGGAGCCUCCUC	406
3009	AAGGGGGGUGCCACACC	168	3009	AAGGGGGGUGCCACACC	168	3027	GGUGGGGCCACGGCCCUU	407
3027	CGGGGACAGGCCGAGCUC	169	3027	CGGGGACAGGCCGAGCUC	169	3045	GAGCUGCGGGCCUGUCCCCG	408
3045	CCAUUUCUUAUUGCCUG	170	3045	CCAUUUCUUAUUGCCUG	170	3063	CAGCGCAUUAAGAAAUGG	409
3063	GCUACCGGUUGACUCCAGG	171	3063	GCUACCGGUUGACUCCAGG	171	3081	CCUGGAAGGUCAACGGUAGC	410
3081	GCACGGGUUGGAAAUAUUC	172	3081	GCACGGGUUGGAAAUAUUC	172	3099	GAUAAUUCUCAACGGUG	411
3099	CACAUUCGUUCUGUGUAUC	173	3099	CACAUUCGUUCUGUGUAUC	173	3117	GAUACAGAAAGCGAUGUG	412
3117	CUCUUUCACAUUUUGCU	174	3117	CUCUUUCACAUUUUGCU	174	3135	AGCAAAACAUUGGAAAGAG	413
3135	UGCUUUUAGGGAUCAGUU	175	3135	UGCUUUUAGGGAUCAGUU	175	3153	AACUGAUCCUCAAUAGCA	414

3153	UUUUUUUUUACAAUGUCA	176	3163	UUUUUUUUUACAAUGUCA	176	3171	UGACAUUUGUAAAACAAAAAA	415
3171	AUAUACUGCCAUAGUACUAG	177	3171	AUAUACUGCCAUAGUACUAG	177	3189	CJAGUACAUAGGCAGUAUAU	416
3189	GUUIUAGUUUUCUUAGA	178	3189	GUUUUAGUUUUCUUAGA	178	3207	UCUAAGAGAAAACUAAAAC	417
3207	AACAUUGUAAUACAGAUGC	179	3207	AACAUUGUAAUACAGAUGC	179	3225	GCAUCUGUAAUACAGUJU	418
3225	CCUUUUUUGUAGUUUUUU	180	3225	CCUUUUUUGUAGUUUUUU	180	3243	AAAAAAACUACAAAAAAAGG	419
3243	UUUUUUUUAUGUGAUCAAU	181	3243	UUUUUUUUAUGUGAUCAAU	181	3261	AUUGAUICACAUAAAAGAAA	420
3261	UUUUUGACUUAUUGUGAUUA	182	3261	UUUUUGACUUAUUGUGAUUA	182	3279	UUAUACAUUAUAGUGACAA	421
3279	ACUGGCUCUAAUCCAAAAG	183	3279	ACUGGCUCUAAUCCAAAAG	183	3297	CJUUUUGGAUAGAGCAGU	422
3297	GGUUGCUGUUCACAAUAC	184	3297	GGUUGCUGUUCACAAUAC	184	3315	GUUAUGUGAAACGCAACC	423
3315	CCUCAUCUUCUACUAGCC	185	3315	CCUCAUCUUCUACUAGCC	185	3333	GCCCCGUUGGUCCACCAUG	424
3333	CAUGGGUGGACCCAGGGGC	186	3333	CAUGGGUGGACCCAGGGGC	186	3351	GCCCCGUUGGUCCACCAUG	425
3351	CAGGUUUCUGCCUGCJUJGG	187	3351	CAGGUUUCUGCCUGCJUJGG	187	3369	CAAAGCAGGCAAAACUG	426
3369	GCGGGCAGACACGGGGCG	188	3369	GCGGGCAGACACGGGGCG	188	3387	GGCUAAGGUAGCAUGAGG	427
3387	GCGAUCUCCACACAGCUGG	189	3387	GCGAUCUCCACACAGCUGG	189	3405	CAGGGGGCGGGGGGGGG	428
3405	GCGGGGGCGGGCCCCGG	190	3405	GCGGGGGCGGGGGGGGG	190	3423	CCUOGGGGGCGGGGGGG	429
3423	GCCGGCGUGGGUGGAAACCG	191	3423	GCCGGCGUGGGUGGAAACCG	191	3441	GGUUCUCAGCCACGGGGC	430
3441	GCGCGCGUGGUCCCCAGAGA	192	3441	GCGCGCGUGGUCCCCAGAGA	192	3459	UCUCUGGGGACACGGGGC	431
3459	ACCAGGGCGUGGUCCCCUU	193	3459	ACCAGGGCGUGGUCCCCUU	193	3477	AAGAGGGGACACAGCCGGU	432
3477	UCUCUCCCCUGGCCUGUG	194	3477	UCUCUCCCCUGGCCUGUG	194	3495	CACAGGGCGAGGGAAAGAGA	433
3495	GAUGGCUGGGCACUCAUCU	195	3495	GAUGGCUGGGCACUCAUCU	195	3513	AGAUGAAAGUGCCAGCAUC	434
3513	UGAUCCGGGGCGGUAGCAUC	196	3513	UGAUCCGGGGCGGUAGCAUC	196	3531	GAUGCUACGCCCCGAUCA	435
3531	CAUAGUAGUUUUUACAGCU	197	3531	CAUAGUAGUUUUUACAGCU	197	3549	AGCUGUAAAACUACUAGU	436
3549	UGUGUUAUUCUUGCGUGU	198	3549	UGUGUUAUUCUUGCGUGU	198	3567	ACACGCAAAAGAAUACACAA	437
3567	UAGCUAUGGAAGUUGCUA	199	3567	UAGCUAUGGAAGUUGCUA	199	3585	UAUGCAACUUCUAGCUA	438
3585	AAUUAUUAUUAUUAUUAU	200	3585	AAUUAUUAUUAUUAUUAU	200	3603	AAUUAUUAUUAUUAUUAU	439
3603	UAUAAACAAAGUGUGCUUAC	201	3603	UAUAAACAAAGUGUGCUUAC	201	3621	GUAGAGACACACUUGUUAU	440
3621	CGUGCCACCCAGGGGUUGU	202	3621	CGUGCCACCCAGGGGUUGU	202	3639	ACAACAGCCGGGGGGCAG	441
3639	UACCUUGGUAGGACUCUAU	203	3639	UACCUUGGUAGGACUCUAU	203	3657	AAUGAGAGGUCCUACGGUA	442
3657	UCGGGAUGAUUGGAAUAGC	204	3657	UCGGGAUGAUUGGAAUAGC	204	3675	GCJAUUUCCAAUCUCCCGA	443
3675	CUUCUGGAAUUUGGUUCAAG	205	3675	CUUCUGGAAUUUGGUUCAAG	205	3693	CJUGAAACAAUUCCCAAAAC	444
3693	GUUUUGGGUAUJUUAUC	206	3693	GUUUUGGGUAUJUUAUC	206	3711	GAUUAACAUACUAAUACAG	445
3711	CUGUUAGUACUAGUGUUC	207	3711	CUGUUAGUACUAGUGUUC	207	3729	GAACACUAGUACAUAAACAG	446
3729	CUGUUAGUACUAGUGUUC	208	3729	CUGUUAGUACUAGUGUUC	208	3747	ACAAAACAAUACAAACAG	447
3747	UAAAUAUACACCAUAGCU	209	3747	UAAAUAUACACCAUAGCU	209	3765	AGCAUUAUUGGUUAUUAU	448
3765	UAAAUAUAAAGAGACUCAA	210	3765	UAAAUAUAAAGAGACUCAA	210	3783	UJGGAGUCUCUUAUUAU	449
3783	AAUCUCAUAGAAGCCAGCU	211	3783	AAUCUCAUAGAAGCCAGCU	211	3801	AGCUGGGCUUCAUUGAGAUU	450

3801	UCACAGUGCUGUGUGCCCC	212	3801	UCACAGUGCUGUGUGCCCC	212	3819	GGGGCACACAGCACUGUGA	451
3819	CGGUACCUAUGCAGCUGC	213	3819	CGGUACCUAUGCAGCUGC	213	3837	GCAGCUUCCUAGGUACCG	452
3837	CCGAACCAAAAGAAUUGC	214	3837	CCGAACCAAAAGAAUUGC	214	3855	GCAAAUUCUUJUGGUUUGG	453
3855	CACCCCGCUGGGGCCAC	215	3855	CACCCCGCUGGGGCCAC	215	3873	GUJGGCCCGAGGGGGUG	454
3873	CGUGGUUGGGCCUGCCC	216	3873	CGUGGUUGGGCCUGCCC	216	3891	GGGCAGGGGCCAACACAG	455
3891	CUGGCAGGGGUCAUCCUG	217	3891	CUGGCAGGGGUCAUCCUG	217	3909	CACAGGAUGGACCCUGCCAG	456
3909	GCUCGGGGGCCAUUCUGGG	218	3909	GCUCGGGGGCCAUUCUGGG	218	3927	CCCGAGAUGGGCCUCCGAGC	457
3927	GCACAGGCCACCCGGCC	219	3927	GCACAGGCCACCCGGCC	219	3945	GGCCGGGGUGGGCCUGUGC	458
3945	CCACCCCUCCAGAACCGG	220	3945	CCACCCCUCCAGAACCGG	220	3963	CCGUUGUUCUGGGGGUGG	459
3963	GCUCACGCCUJACCUAAC	221	3963	GCUCACGCCUJACCUAAC	221	3981	GGUJGAGGUAGCGGUAGGC	460
3981	CAUCCUGGGCUGGGCUCU	222	3981	CAUCCUGGGCUGGGCUCU	222	3999	AGACGCCGAGCCAGGAUG	461
3999	UGUCUGAACCCACGGGG	223	3999	UGUCUGAACCCACGGGG	223	4017	CCCCCGCUGGUUICAGACA	462
4017	GCCUUGAGGGACGUUUUU	224	4017	GCCUUGAGGGACGUUUUU	224	4035	ACAAAGCCUCCUAAAGGC	463
4035	UCUGUCGUAGGGGCAAG	225	4035	UCUGUCGUAGGGGCAAG	225	4053	CUUGCCCCAUACCGACAGA	464
4053	GGGCACAAAGUCCUGGAUGU	226	4053	GGGCACAAAGUCCUGGAUGU	226	4071	ACAUCCAGGACUUGGUGCC	465
4071	UUGUGUJUAUCGAGGGCC	227	4071	UUGUGUJUAUCGAGGGCC	227	4089	GCCUCUCUGGUACACACAA	466
4089	CAAAGGCUGGGCAAGUG	228	4089	CAAAGGCUGGGCAAGUG	228	4107	CAUCUGCCACCCGUUUUG	467
4107	GCACGGGGCACAGGGAGU	229	4107	GCACGGGGCACAGGGAGU	229	4125	ACUCGGCUGGUUGCCCCUGC	468
4125	UCUGUCCUGUGACGCCAA	230	4125	UCUGUCCUGUGACGCCAA	230	4143	UGGCGCUGUACAGGACAGA	469
4143	AGUCUGAGGGGUUGGGCGG	231	4143	AGUCUGAGGGGUUGGGCGG	231	4161	CGCCCAAGACCCUCAGACU	470
4161	GGGGGGGGCUGGGCUGUG	232	4161	GGGGGGGGCUGGGCUGUG	232	4179	CACAGCCACAGCCGCCGC	471
4179	GCAUJUUCUGGUUGACCGC	233	4179	GCAUJUUCUGGUUGACCGC	233	4197	GGGGUGCAACCAAGAACUGC	472
4197	CGGGCUUCCAGCACCAA	234	4197	CGGGCUUCCAGCACCAA	234	4215	UUGGGUGGUAGGCGCCGC	473
4215	ACAUGUAAACGGCAUGUU	235	4215	ACAUGUAAACGGCAUGUU	235	4233	AAACAUUGCCGGGUUACAUU	474
4233	UCCAGCAGAAAGACAAAAAG	236	4233	UCCAGCAGAAAGACAAAAAG	236	4251	CUUUUUCUUCUGGUUGGA	475
4251	GACAACAUAAAAGGUUCUAG	237	4251	GACAACAUAAAAGGUUCUAG	237	4269	CUAGACUUCGUUUUUAUUC	476
4269	AAAAAAAACUGGUAAAAC	238	4269	AAAAAAAACUGGUAAAAC	238	4287	GUUUUACAGGUUUUUAUUC	477
4286	ACCCCAAAAAAA	239	4286	ACCCCAAAAAAA	239	4304	UUUUUUUUUUUUUGGGU	478

The 3'-ends of the Upper sequence and the Lower sequence of the siNA construct can include an overhang sequence, for example about 1, 2, 3, or 4 nucleotides in length, preferably 2 nucleotides in length, wherein the overhanging sequence of the lower sequence is optionally complementary to a portion of the target sequence. The upper sequence is also referred to as the sense strand, whereas the lower sequence is also referred to as the antisense strand. The upper and lower sequences in the Table can further comprise a chemical modification having Formulae I-VII or any combination thereof.

Table III: cyclin D1 Synthetic Modified siRNA constructs

Target Pos	Target	SeqID	RPI #	Allases	Sequence	SeqID
693	GAACACUUCUCUCCAAAUGCC	479	31009	CCND1:695U21 siRNA sense	ACACUUCUCUCCAAAUGTT	483
1626	GCUGUAGUGGGGUUCUAGGCAUC	480	31010	CCND1:1628U21 siRNA sense	UGUAGUGGGGUUCUAGGCAATT	484
2615	ACACACAAACCUUCUCCUUIUGA	481	31011	CCND1:2617U21 siRNA sense	ACACAAACCUUCUCCUUIUGU	485
3122	UCACAUUUUUGUGUGCUUAGGGA	482	31012	CCND1:3124U21 siRNA sense	ACAUUUUUGUGUGCUUAGGTT	486
693	GAACACUUCUCUCCAAAUGCC	479	31085	CCND1:713L21 siRNA (695C) antisense	CAUUGGAGAGGAAGUGU	487
1626	GCUGUAGUGGGGUUCUAGGCAUC	480	31086	CCND1:2635U21 siRNA (2617C) antisense	UGCCUAGAACCCACUACATT	488
2615	ACACACAAACCUUCUCCUUUGA	481	31087	CCND1:2635U21 siRNA (2617C) antisense	AAAGGCAGAAAGGUUUGUGU	489
3122	UCACAUUUUUGUGUGCUUAGGGA	482	31088	CCND1:3142L21 siRNA (3124C) antisense	CAAUAGCAGCAAACAAUGU	490
693	GAACACUUCUCUCCAAAUGCC	479	31304	CCND1:695U21 siRNA stab04 sense	B AcACUUCUCCUCCAAAUGTT	491
1626	GCUGUAGUGGGGUUCUAGGCAUC	480	30746	CCND1:1628U21 siRNA stab04 sense	B uGUAGUGGGGuuucuAGGcATT	492
2615	ACACACAAACCUUCUCCUUUGA	481	30747	CCND1:2617U21 siRNA stab04 sense	B AcACAAACCUUCUCCUUuTT	493
3122	UCACAUUUUUGUGUGCUUAGGGA	482	30748	CCND1:3124U21 siRNA stab04 sense	B AcAuuGuuuGcuGcuAuuGTT	494
693	GAACACUUCUCUCCAAAUGCC	479	31305	CCND1:713L21 siRNA (695C) stab05 antisense	cAuuuuGGAGGGAAAGuGuTsT	495
1626	GCUGUAGUGGGGUUCUAGGCAUC	480	30750	CCND1:1646L21 siRNA (1628C) stab05 antisense	uGcuuACAAACCCACuACATsT	496
2615	ACACACAAACCUUCUCCUUUGA	481	30751	CCND1:2635L21 siRNA (2617C) stab05 antisense	AAAGGcAGAAGGuuGuGuTsT	497
3122	UCACAUUUUUGUGUGCUUAGGGA	482	30752	CCND1:3142L21 siRNA (3124C) stab05 antisense	cAAuAGGcAGCAAACAAUGuTsT	498
693	GAACACUUCUCUCCAAAUGCC	479	31307	CCND1:695U21 siRNA stab07 sense	B AcACUUCUCCUCCAAAAGuTsT	499
1626	GCUGUAGUGGGGUUCUAGGCAUC	480	31308	CCND1:1628U21 siRNA stab07 sense	B uGuAGUGGGGuuucuAGGcATT	500
2615	ACACACAAACCUUCUCCUUUGA	481	31309	CCND1:2617U21 siRNA stab07 sense	B AcACAAACCUUCUCCUUuTT	501
3122	UCACAUUUUUGUGUGCUUAGGGA	482	31310	CCND1:713L21 siRNA (695C) stab11 antisense	B AcAuuGuuuGcuGcuAuuGTT	502
693	GAACACUUCUCUCCAAAUGCC	479			cAuuuuGGAGGGAAAGuGuTsT	503

1626	GCUGUAGUGGGGUUCUAGGCAUC	480	CCND1:1646L21 sirNA (1628C) stab11 antisense	uGCCUAGAACCCAcuACATsT	504	
2615	ACACACAAACCUUUCUGCCUUUGA	481	CCND1:2635L21 sirNA (2617C) stab11 antisense	AAAGGGAGAAGGGuuuGuGuTsT	505	
			CCND1:3142L21 sirNA (3124C) stab11 antisense	cAAuAGcAGcAAAcAAuGuTsT	506	
3122	UCACAUUUGUUGCUGCUAUUGGA	482	CCND1:685U21 sirNA inv stab04 sense	B GUAAAACccucuuuAcATT B	507	
693	GAACACUUUCUCUCCAAA AUGCC	479	31316	CCND1:713L21 sirNA (695C) inv stab05 antisense	uGuGAAGGAGAGGGuuuActsT	508
693	GAACACUUUCUCUCCAAA AUGCC	479	31317			

Uppercase = ribonucleotide

u,c = 2'-deoxy-2'-fluoro U, C

T = thymidine

B = inverted deoxy abasic

s = phosphorothioate linkage

A = deoxy Adenosine

G = deoxy Guanosine

Table IV

Non-limiting examples of Stabilization Chemistries for chemically modified siRNA constructs

Chemistry	pyrimidine	Purine	cap	p=S	Strand
“Stab 1”	Ribo	Ribo	-	5 at 5'-end 1 at 3'-end	S/AS
“Stab 2”	Ribo	Ribo	-	All linkages	Usually AS
“Stab 3”	2'-fluoro	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
“Stab 4”	2'-fluoro	Ribo	5' and 3'-ends	-	Usually S
“Stab 5”	2'-fluoro	Ribo	-	1 at 3'-end	Usually AS
“Stab 6”	2'-O-Methyl	Ribo	5' and 3'-ends	-	Usually S
“Stab 7”	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
“Stab 8”	2'-fluoro	2'-O-Methyl	-	1 at 3'-end	Usually AS
“Stab 9”	Ribo	Ribo	5' and 3'-ends	-	Usually S
“Stab 10”	Ribo	Ribo	-	1 at 3'-end	Usually AS
“Stab 11”	2'-fluoro	2'-deoxy	-	1 at 3'-end	Usually AS

CAP = any terminal cap, see for example **Figure 10**.

All Stab 1-11 chemistries can comprise 3'-terminal thymidine (TT) residues

All Stab 1-11 chemistries typically comprise 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

Table V

A. 2.5  $\mu$ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 $\mu$ L	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 $\mu$ L	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 $\mu$ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 $\mu$ L	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 $\mu$ L	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2  $\mu$ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 $\mu$ L	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 $\mu$ L	45 sec	233 min	465 sec
Acetic Anhydride	655	124 $\mu$ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 $\mu$ L	5 sec	5 sec	5 sec
TCA	700	732 $\mu$ L	10 sec	10 sec	10 sec
Iodine	20.6	244 $\mu$ L	15 sec	15 sec	15 sec
Beaucage	7.7	232 $\mu$ L	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2  $\mu$ mol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 $\mu$ L	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 $\mu$ L	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 $\mu$ L	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 $\mu$ L	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 $\mu$ L	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 $\mu$ L	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 $\mu$ L	NA	NA	NA

5 • Wait time does not include contact time during delivery.

• Tandem synthesis utilizes double coupling of linker molecule

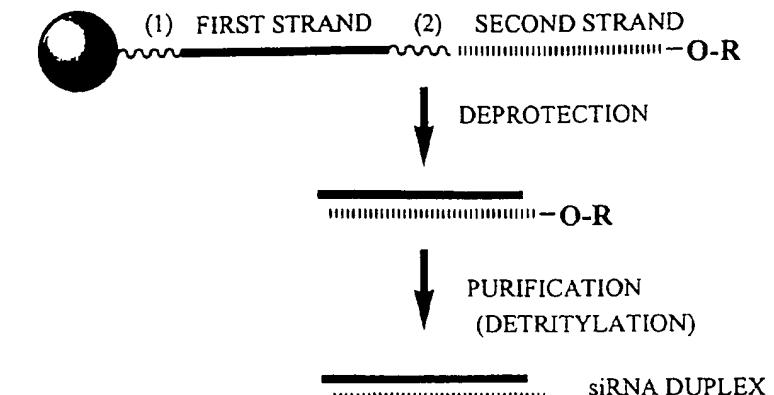
CLAIMS

What we claim is:

1. A short interfering nucleic acid (siNA) molecule that down-regulates expression of one or more cyclin genes by RNA interference.
- 5 2. The siNA molecule of claim 1, wherein the cyclin gene is cyclin D1.
3. The siNA molecule of claim 1, wherein said siNA molecule comprises no ribonucleotides.
4. The siNA molecule of claim 1, wherein said siNA molecule comprises ribonucleotides.
- 10 5. The siNA molecule of claim 1, wherein said siNA molecule is double stranded.
6. The siNA molecule of claim 5, wherein said siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein, and wherein said siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a cyclin D1 gene or a portion thereof.
- 15 7. The siNA molecule of claim 6, wherein said antisense strand and said sense strand each comprise about 19 to about 29 nucleotides, and wherein said antisense strand and said sense strand share at least about 19 complementary nucleotides.
8. The siNA molecule of claim 5, wherein said siNA molecule comprises an antisense 20 region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a cyclin D1 gene or a portion thereof.
9. The siNA molecule of claim 8, wherein said antisense region and said sense region 25 each comprise about 19 to about 29 nucleotides, and wherein said antisense region and said sense region share at least about 19 complementary nucleotides.
10. The siNA molecule of claim 1, wherein said siNA molecule is single stranded.

11. The siNA molecule of claim 10, wherein said siNA comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein.
12. The siNA molecule of claim 11, wherein said siNA molecule comprises a sequence having about 19 to about 29 nucleotides.
13. The siNA molecule of claim 1, wherein said siNA molecule comprises a sense region and an antisense region and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a cyclin D1 protein and said sense region comprises a nucleotide sequence complementary to said antisense region.
14. The siNA molecule of claim 1, wherein said siNA molecule is assembled from two oligonucleotide fragments wherein one fragment comprises a sense region and a second fragment comprises an antisense region of said siNA molecule.
15. The siNA molecule of claim 13, wherein said sense region and said antisense region comprise separate oligonucleotides.
16. The siNA molecule of claim 13, wherein said sense region and said antisense region are connected via a linker molecule.
17. The siNA molecule of claim 16, wherein said linker molecule is a polynucleotide linker.
20. 18. The siNA molecule of claim 16, wherein said linker molecule is a non-nucleotide linker.
19. The siNA molecule of claim 13, wherein said sense region comprises a 3'-terminal overhang and said antisense region comprises a 3'-terminal overhang.
20. 25. The siNA molecule of claim 19, wherein said 3'-terminal overhangs each comprise about 2 nucleotides.
21. The siNA molecule of claim 19, wherein the 3'-terminal overhang of the antisense region is complementary to RNA encoding a cyclin D1 protein.

22. The siNA molecule of claim 13, wherein said sense region comprises one or more 2'-O-methyl pyrimidine nucleotides and one or more 2'-deoxy purine nucleotides.
23. The siNA molecule of claim 13, wherein any pyrimidine nucleotides present in said sense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein any purine nucleotides present in said sense region comprise 2'-deoxy purine nucleotides.  
5
24. The siNA molecule of claim 19, wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.
25. The siNA molecule of claim 13, wherein said sense region comprises a 3'-end and a 5'-end, and wherein a terminal cap moiety is present at the 5'-end, the 3'-end, or both  
10 of the 5' and 3' ends of said sense region.
26. The siNA molecule of claim 25, wherein said terminal cap moiety is an inverted deoxy abasic moiety.
27. The siNA molecule of claim 13, wherein said antisense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine  
15 nucleotides.
28. The siNA molecule of claim 13, wherein any pyrimidine nucleotides present in said antisense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein any purine nucleotides present in said antisense region comprise 2'-O-methyl purine nucleotides.
- 20 29. The siNA molecule of claim 19, wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.
30. The siNA molecule of claim 28, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.
31. The siNA molecule of claim 13, wherein said antisense region comprises a glyceryl  
25 modification at the 3' end of said antisense region.
32. The siNA molecule of claim 19, wherein said 3'-terminal overhangs comprise deoxyribonucleotides.

**Figure 1**

= SOLID SUPPORT

R = TERMINAL PROTECTING GROUP

FOR EXAMPLE:

DIMETHOXYSITYL (DMT)

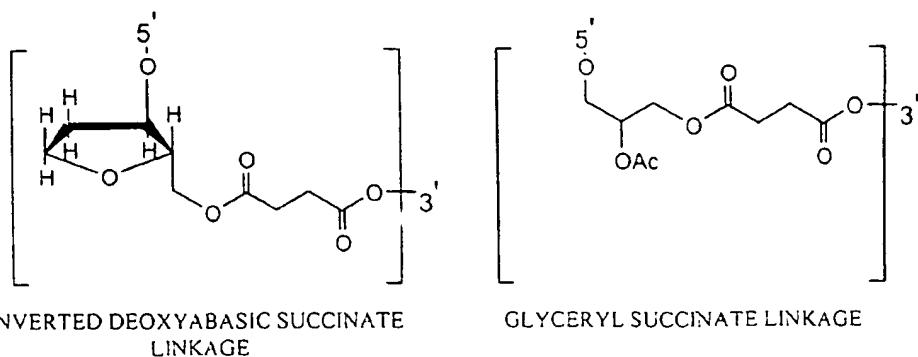
(1)

= CLEAVABLE LINKER

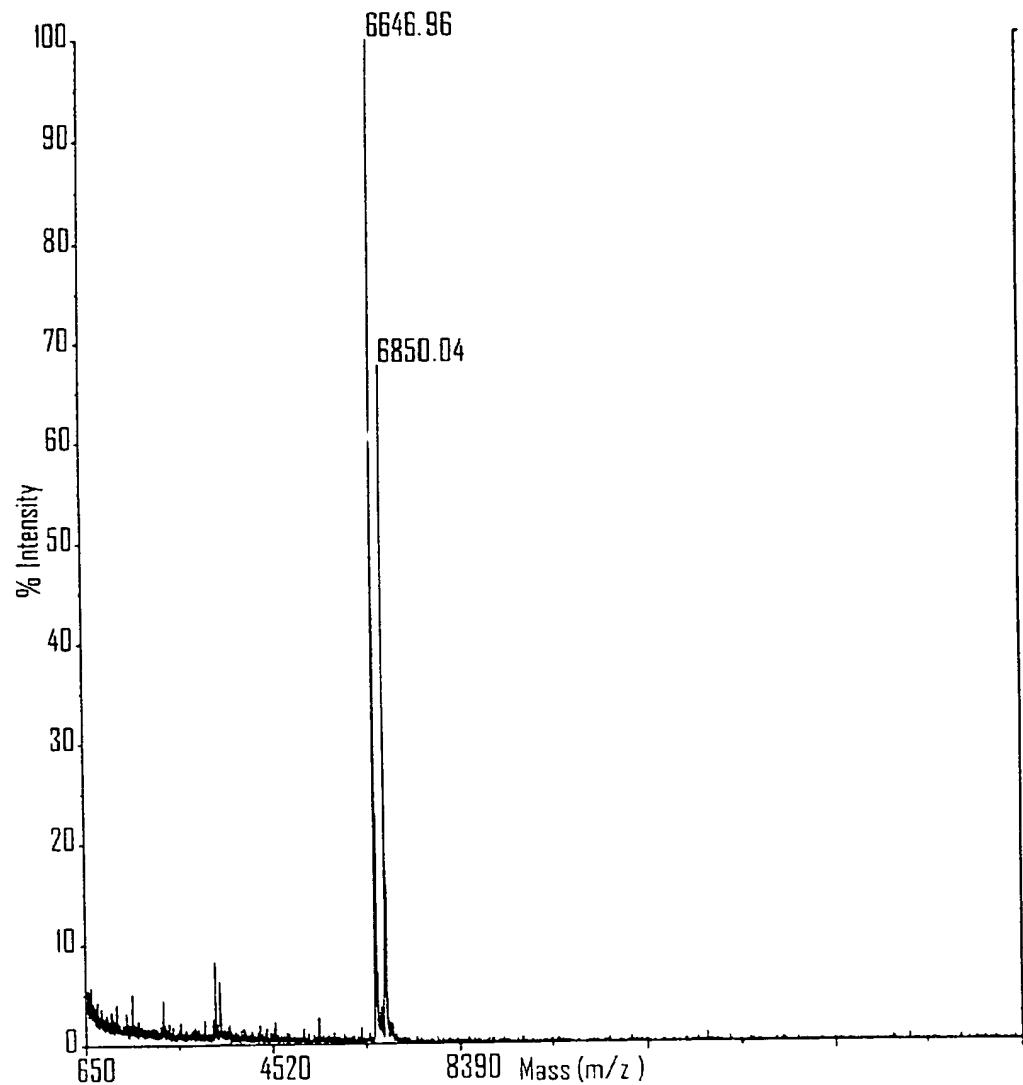
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR  
INVERTED DEOXYABASIC SUCCINATE)

(2)

= CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR  
INVERTED DEOXYABASIC SUCCINATE)

*Figure 2*



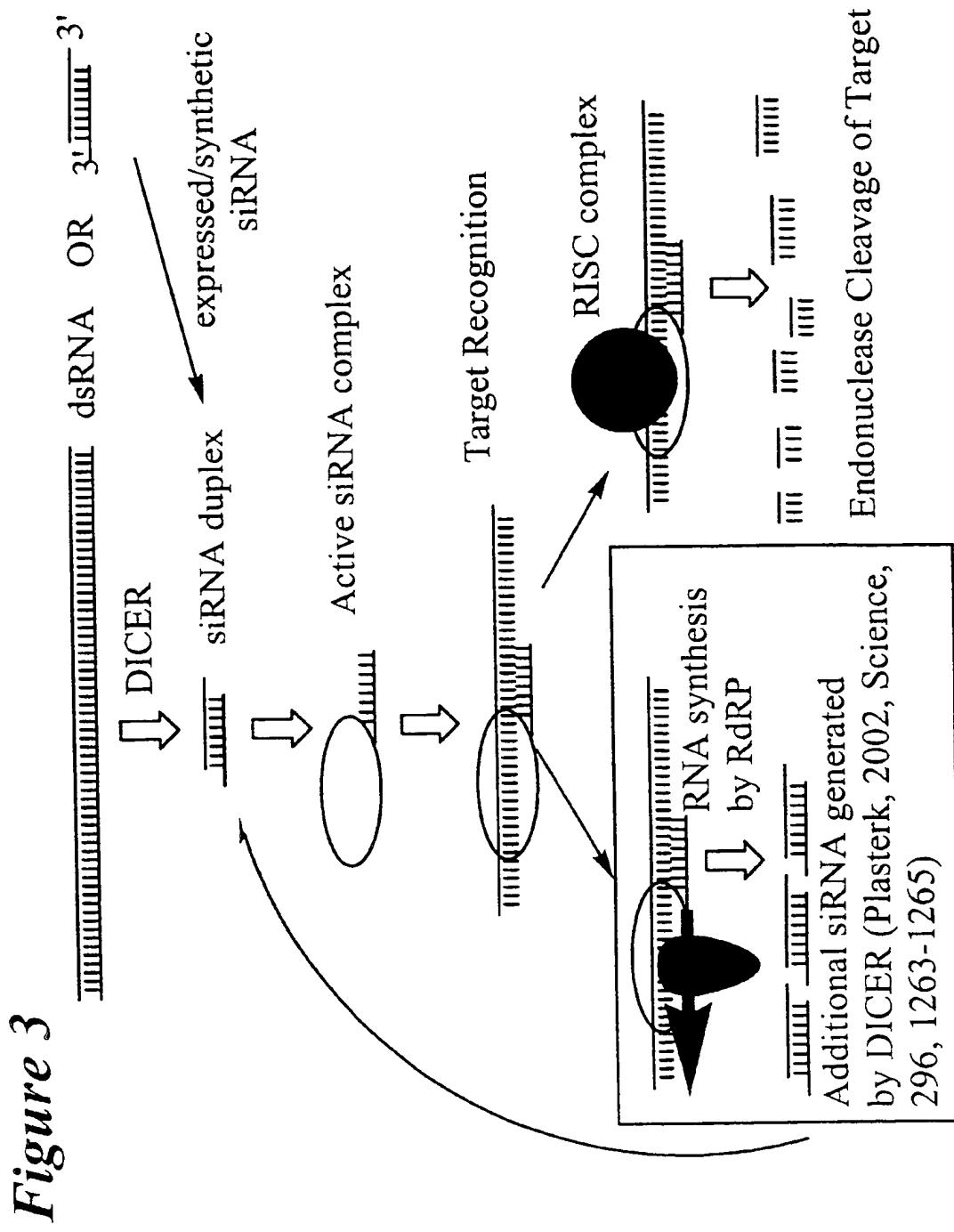
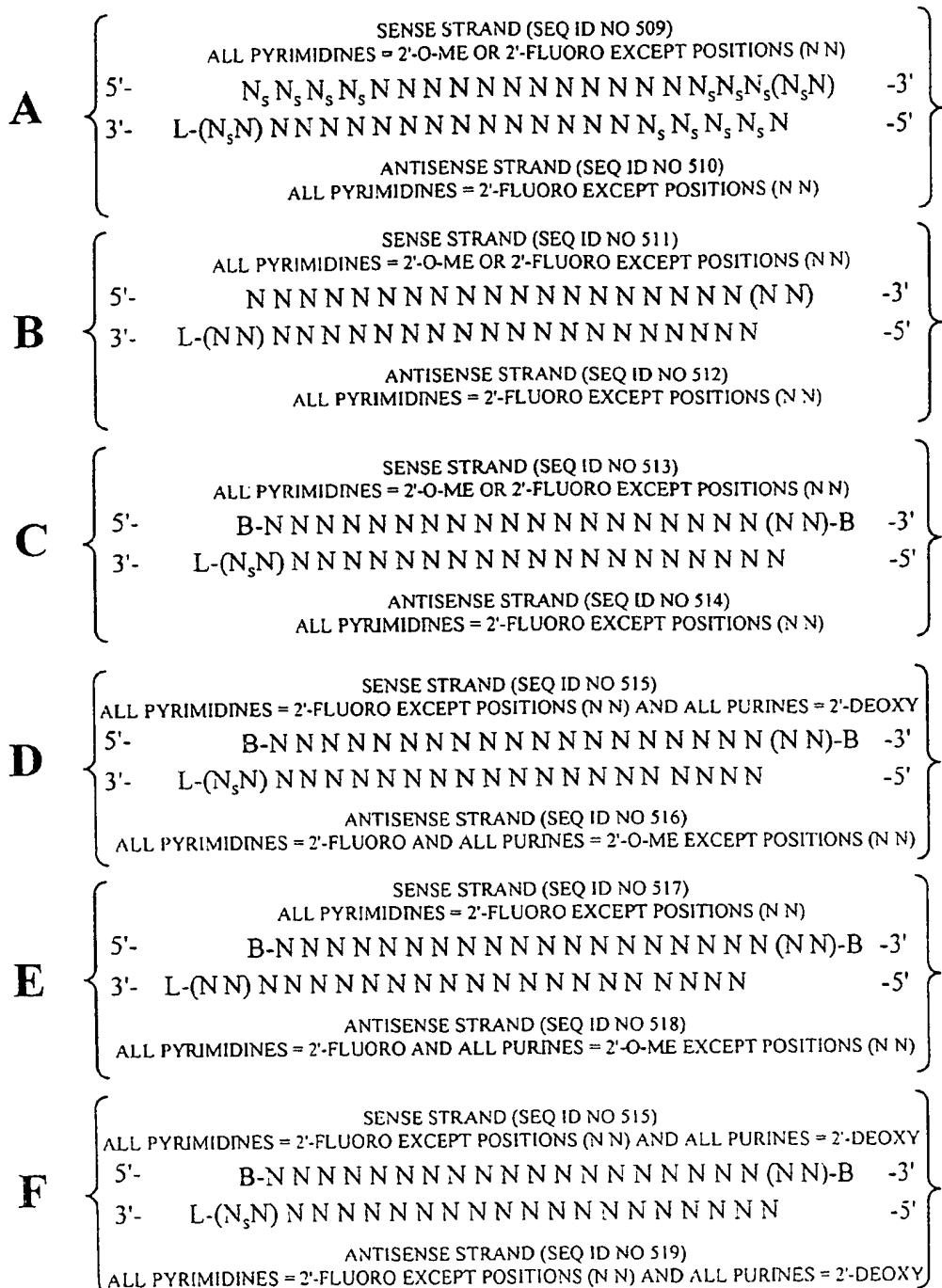


Figure 4



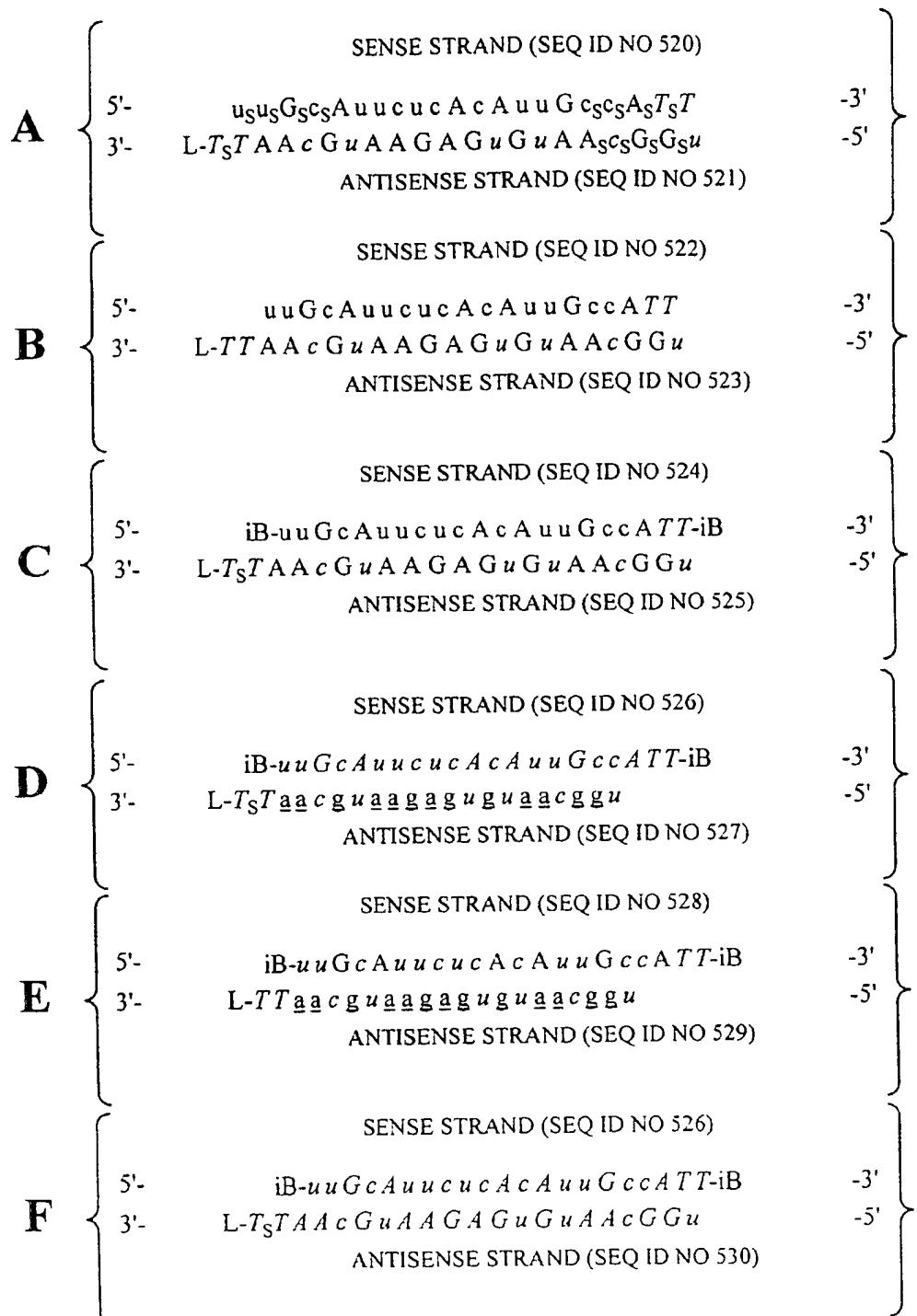
POSITIONS (NN) CAN COMprise ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE) OR UNIVERSAL BASES

(eg. THYMIDINE) OR UNIVERSAL BASES  
B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP  
THAT IS OPTIONAL LY PRESENT

**L = GLYCERYL MOIETY THAT IS OPTIONALY PRESENT**

L = GLYCERYL MOIETY THAT IS OPTIONALLY PRESENT  
S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE

## Figure 5



lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro

*italic lower case* = 2'-deoxy-2'-fluoro

underline = 2'-O-methyl

*ITALIC UPPER CASE* = DEOXY

B = INVERTED DEOXYABASIC

L - GLYCERYL MOIETY OPTIONAL PRESENT

S = PHOSPHOROTHIOATE OR

PHOSPHORODITHIOATE

Figure 6

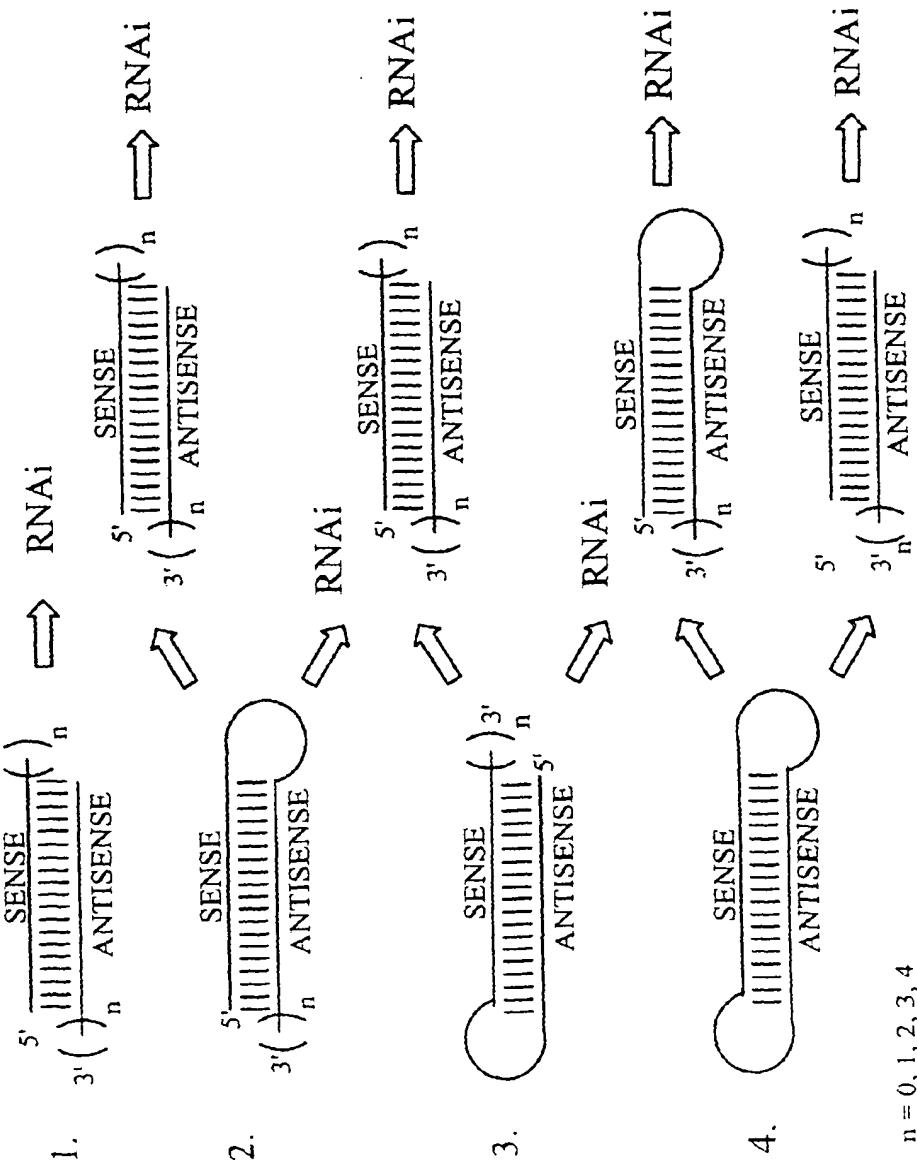
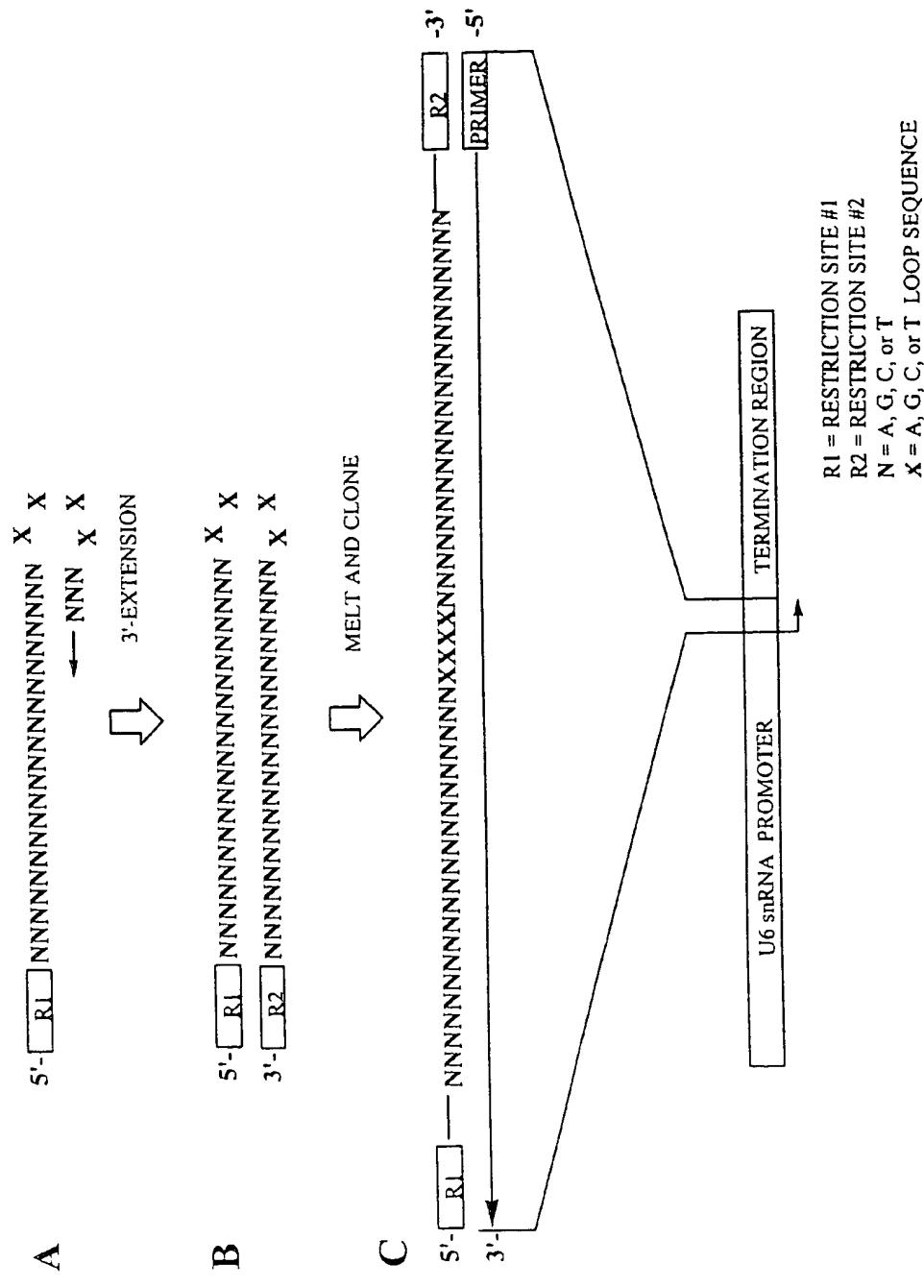
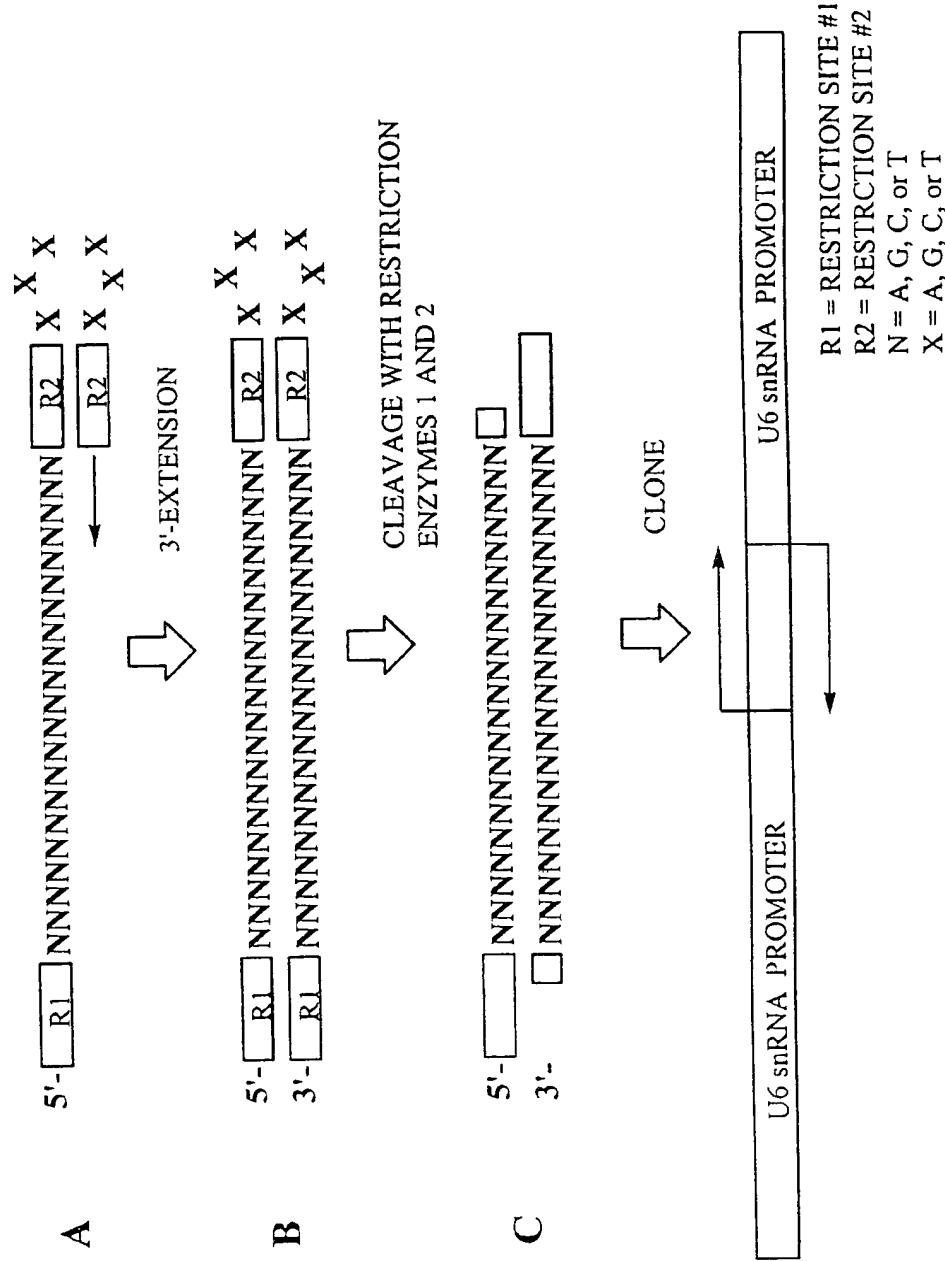


Figure 7



*Figure 8*

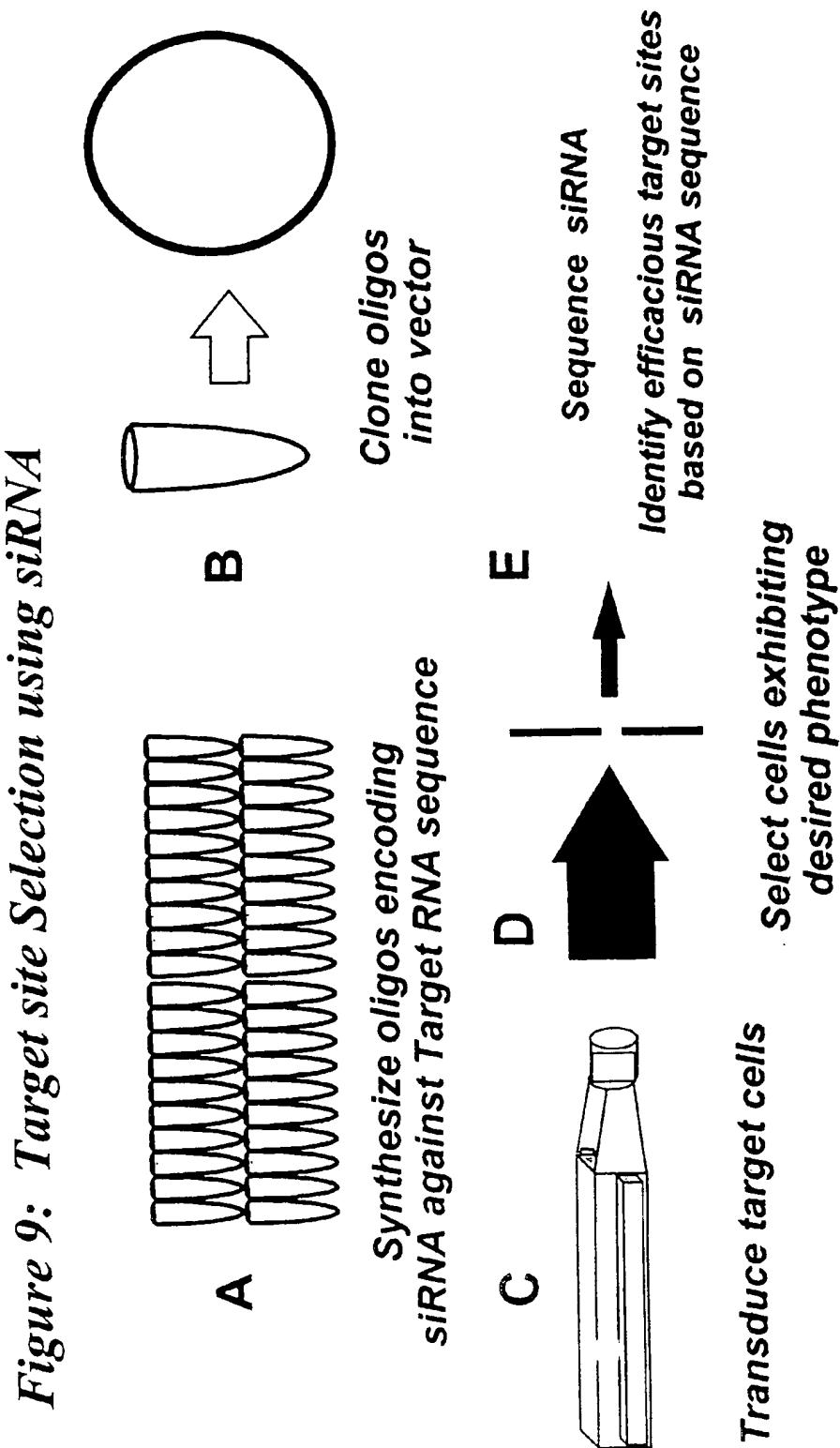
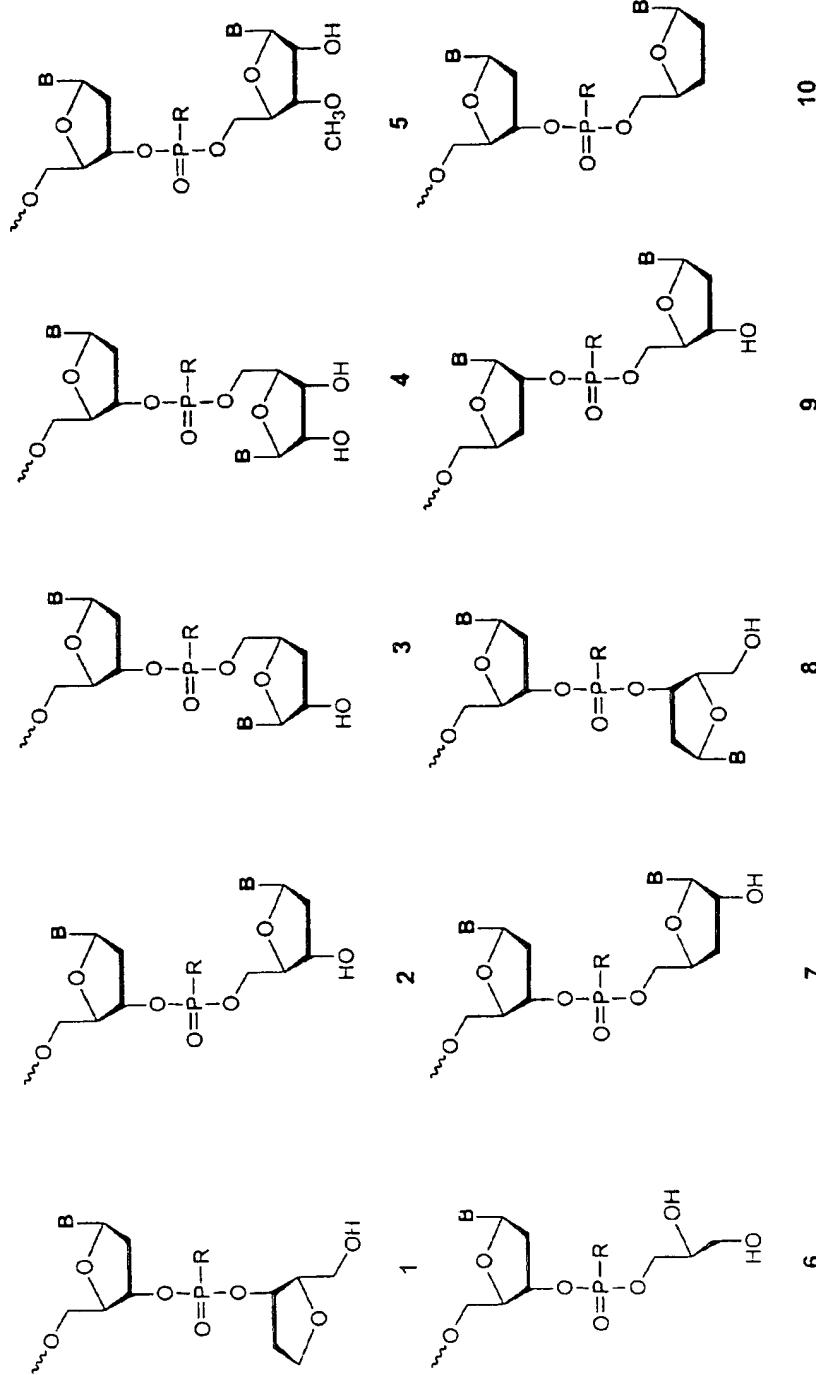
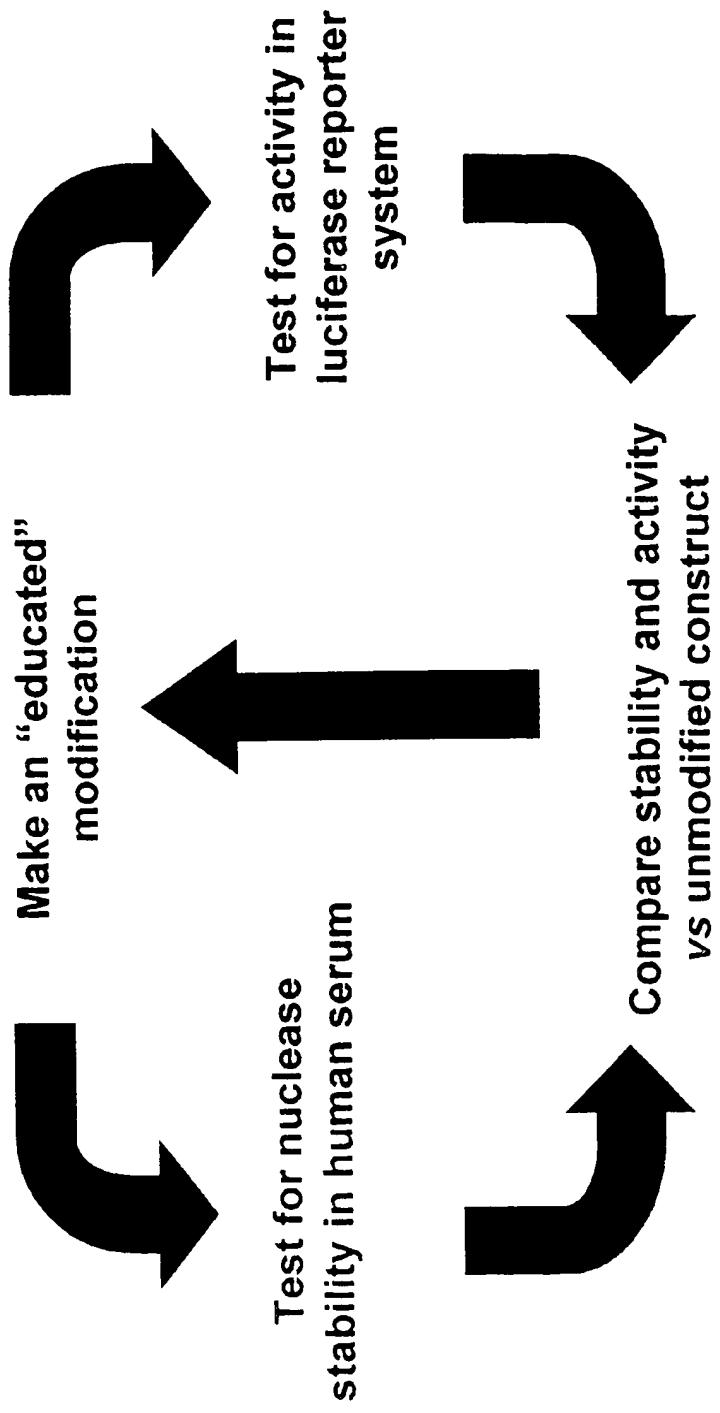


Figure 10



R = O, S, N, alkyl, substituted alkyl, O-alkyl, alkaryl, or aralkyl  
 B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

*Figure 11: Modification Strategy*



**Figure 12: A549 24h CCND1 mRNA Expression**

